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Phylogeography of *Androctonus* scorpions from the Maghreb Region

por
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Abstract

Venomous animals, such as scorpions, have always evoked fascination to mankind. DNA sequence data has become the most used molecular data in estimating evolutionary history and scorpion researchers have started to use these tools to understand the phylogenetic history of this group that was previously difficult to ascertain. In this thesis, mtDNA markers were used to produce the first multi-gene (COI, 16S and 12S) phylogeny of *Androctonus* scorpions. A total of 110 new sequences from six species were used to investigate phylogeographical patterns in North Africa using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The study also produced the first sequence data for two species and the first sequences for specimens from three countries. In addition, the geographic sampling coverage of *Androctonus* was greatly enhanced with unreported locations, confirming former conjectures regarding their range. In the analysis of *Androctonus*, high levels of genetic diversity were found within 13 well-resolved clades that also presented geographical coherence. The bulk of the diversity in the Maghreb is found in Morocco, where this study shows a greater level of cryptic variation than was previously identified. The level of pairwise genetic distances between endemic clades within Morocco can be as high as the distance between clades occurring thousands of kilometers away in other parts of North Africa. In Tunisia this study corroborates a phylogeographical split in *A. australis* found in earlier studies, and shows the two separated clades extend well beyond Tunisia. Scorpion venom is known to vary regionally, even within single species. *Androctonus* are highly poisonous scorpions and studies identifying regional diversity, such as presented here, can have direct application in developing therapeutic measures. Additionally, a molecular phylogeny of the Scorpiones order was produced. 20 species of seven scorpion families were sequenced for three mitochondrial genes (12S, 16S and COI). This phylogeny was part of a study that focused on the scorpion's pincers (pedipalps), particularly on the influence that different cuticular shapes have on the pinching performance. The phylogeny was used to account for the phylogenetic signal in the studied traits by a technique called Independent Contrasts. In that study, we further assessed the evolution of the performance of morphological variants, delimiting groups with similar shapes and testing their performance *in silico* under natural loading condition. Our work is valuable for identifying DNA markers that are informative in scorpion phylogenetic estimate both at the species and family levels. More importantly, these results will hopefully lead on to future integrative studies uniting distinct areas such as toxicology and functional morphology, propelling our knowledge of these animals.

Resumo

Os animais venenosos, como são exemplo os escorpiões, desde sempre suscitaram fascínio ao Homem. A sequenciação do ADN é a mais importante fonte de informação utilizada em biologia evolutiva, no entanto o seu uso na reconstrução da filogenia dos escorpiões encontra-se ainda a dar os primeiros passos. Na presente tese, foram usados marcadores de mtDNA para construir a primeira filogenia multi-gene (COI, 16S e 12S) em escorpiões *Androctonus*. Foi usado um total de 110 novas sequências provenientes de seis espécies para examinar padrões filogeográficos no Norte de África usando as metodologias de Máxima Verosimilhança e Inferência Baiesiana. Do estudo resultaram também as primeiras sequências para duas espécies bem como para espécimes originários de três países inexplorados para este género de escorpião. Adicionalmente, a amostragem de *Androctonus* efetuada ampliou largamente a área de distribuição conhecida de várias espécies no Magrebe, confirmando antigas suposições em relação à sua distribuição. Na análise dos *Androctonus* foram encontrados 13 clados que apresentam suporte significativo e coerência morfológica e geográfica. O maior volume de diversidade genética no Magrebe foi encontrado em Marrocos, pais onde se encontrou variação críptica superior à esperada. Entre clados endémicos de Marrocos o nível de *distância genética entre pares* pode apresentar-se tão elevado quanto as distâncias genéticas entre clados que distam milhares de quilómetros de distância entre si no Norte de África. Na Tunísia, este estudo corroborou a presença de uma divisão filogeográfica em *A. australis* encontrada em estudos anteriores e demonstrou que estes clados se estendem muito além deste país. É conhecido que o veneno de escorpião varia regionalmente, mesmo intra-especificamente. Os escorpiões do género *Androctonus* são altamente venenosos e estudos que identifiquem diversidade regional, como aqui se apresenta, podem ter aplicação direta no desenvolvimento de medidas terapêuticas no tratamento de casos de escorpionismo. Adicionalmente, foi produzida uma filogenia a nível da ordem Scorpiones. Foram sequenciados três genes mitocondriais a partir de 20 espécies provenientes de sete famílias de escorpião. Esta filogenia fez parte dum estudo focado nas chelas dos pedipalpos do escorpião (pedipalpos), em particular na influência que diferentes formas da cutícula apresentam sobre a sua *performance* de aperto. Esta filogenia foi usada para acomodar o sinal filogenético presente nas características estudadas através da técnica designada por *Independent Contrasts*. Neste estudo abordamos igualmente a evolução da *performace* de variantes morfológicas, delimitando grupos com formas semelhantes e testando o seu desempenho sob condições de carga naturais *in silico*.

O nosso trabalho é útil na identificação de marcadores de DNA informativos para o cálculo de filogenias de escorpião aos níveis de família e espécie. Mais importantes ainda, estes resultados desbloquearão futuros estudos integrativos, unindo áreas de investigação distintas, como por exemplo a toxicologia e a morfologia funcional, impulsionando o nosso conhecimento destes animais.

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Thematic Organization

The thesis is divided in four chapters:

Chapter I starts with the “Introduction”, which provides a background of scorpion biology in general. The main goals are also given in this chapter under the heading “Objectives”;

Chapter II begins with a subchapter entitled “Preface” which provides a detailed view regarding *Androctonus* scorpions and the subject’s relevance as well as its impact. Then, the main dataset developed during the thesis program is presented in the form of a scientific paper;

Chapter III presents a journal article produced in co-authorship with Dr. Arie van der Meijden and collaborators. As in Chapter II the article is preceded by an introduction into the subject

The last chapter, **Chapter IV** gives the closing statements, future work perspectives and the author’s personal remarks as well.

The **References** section contains all citations quoted in the thesis except those cited in the articles.

Chapter I

General Introduction

Objectives

1.1 General Introduction

1.1.1 State of the Art

Scorpions are a primitive and fascinating group of animals. Since early human societies scorpions have been the object of worship and fear as well. This negative reputation is built around myths and popular beliefs but also because few scorpions are, in fact, capable of causing human death. Their appearance as aquatic organisms dates back 450 million years but it was only upon transition to land that they experienced a great radiation in the family tree (Polis, 1990). A unique set of biological features allowed them to colonize all continents (except for Antarctica) and environments. These nocturnal predators are abundant in tropical forests and even in elevated mountains but the bulk of genera diversity occurs in arid and desert environments (Brownell and Polis, 2001).

Scientific research concerning scorpion biology is an integrative field, combing aspects of physiology, behavior, ecology and evolutionary biology. Today, as an outcome, scorpions are an arachnid model to investigate broader questions of organismal biology.

1.1.2 Origins and Biology

Scorpions are an old lineage of terrestrial arthropods and one of the most cosmopolitan orders of the Arachnida class after spiders (Aranae), mites and ticks (Acari), pseudoscorpions (Pseudoscorpiones) and opiliones (Opiliones Sundevall, 1833) (Savory, 1977). They first appear in the fossil record in the middle Silurian (around 425 – 450 million years ago). They are a sister group to opiliones and together form a taxonomic grouping named Stomothecata. The position Stomothecata occupy in the Arachnid class is still debated but might be a sister clade to all the other subclasses (Shultz, 2007). Ecologically there is also debate if scorpions shared the aquatic setting with Eurypterids, a group of arthropods that was thought to be closely related do scorpions (Gess, 2013; Legg et al., 2013). Around the middle Carboniferous (250 – 300 million years), the fossil record starts to show the first terrestrial scorpions (Polis and Sissom, 1990) (Figure 1). Today, there are almost 2000 extant species in the Scorpiones order (extinct species are not inserted in the scope of this work) (Rein, 2012). Nevertheless, it constitutes a minor percentage of all arachnid species, circa 1,6 % (Hallan, 2005).

There are two key phases in scorpion evolution: the aquatic-to-inland transition and colonization of land. On one hand, the shift to land represented great morphological changes that impacted directly on the role as terrestrial predators. On the other hand, taking over land-masses produced a quick radiation into more than 50 families (Brownell and Polis, 2001). Surprisingly, this genetic outburst was not followed by major morphological modifications, unlike insects (Hexapoda, Insecta) and the most abundant arachnids (ticks and spiders). Although most families from that initial radiation are extinct, they were much like the present species morphologically (Brownell and Polis, 2001). Although there are ecomorphological adaptations, these do not obscure the general resemblance among scorpions.

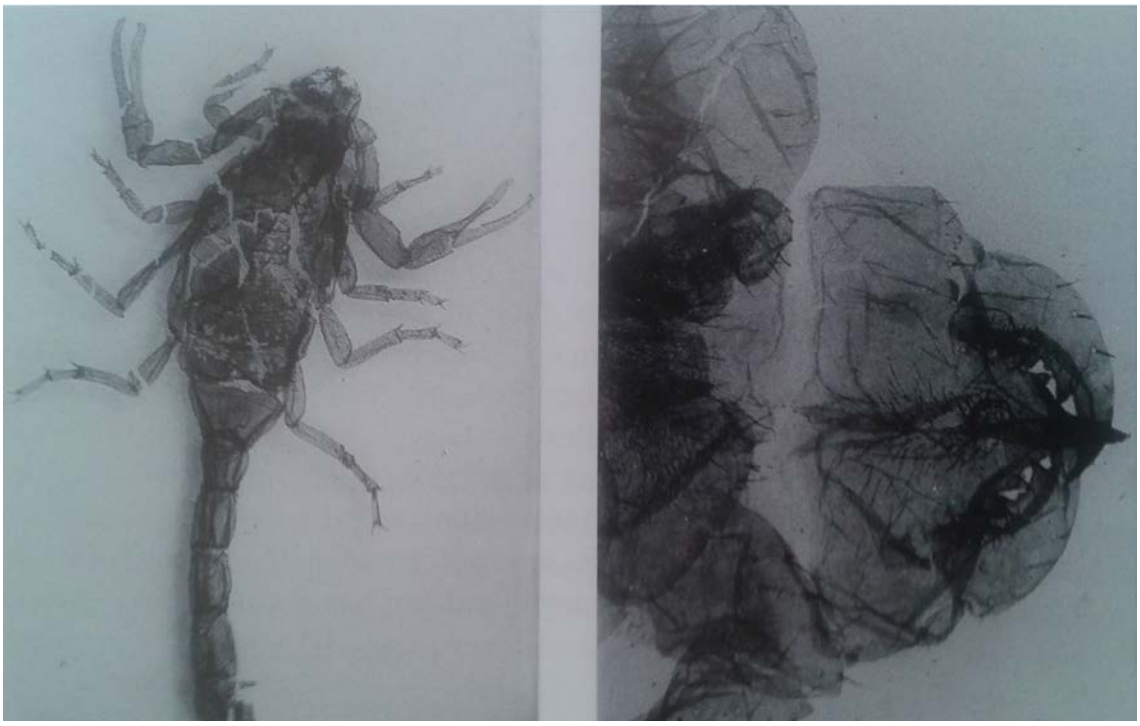


Figure 1: The first known terrestrial scorpions; Lower Carboniferous scorpions from the East Kirkton Quarry near Edinburgh, Scotland. To the left, a fourth stadium juvenile mesoscorpion. To the right, a detail of chelicerae, proximal end of pedipalps, and anterior end of oral tube (From Jeram, 2000).

Apart from polar areas, scorpions exist everywhere. They dwell in the highly eroded intertidal coast of Baja California, the Ural and Caucasus elevated mountains and even strictly in caves. In these habitats, some are specialized burrowers, whereas others are vagrant, saxicolous or arboreal (Lamoral, 1979; Polis, 1990). Two environments are, however, of top position considering genetic diversity. When it comes to species diversity, tropical forests are home to the largest number of scorpions. On the other hand, desert and arid landscapes harbor most of the extant

genera. It is not a coincidence that scorpions are customarily associated with deserts. In such habitats, scorpions appear to be one of the most successful and important predators in terms of density, diversity and biomass (Polis and Sissom, 1990). They are not only widely spread, but they are ecologically essential in certain communities. They can reach population densities of 5000 - 10000 animals per hectare, exceeded only by ants and termites (Brownell and Polis, 2001). Desert species can endure temperatures several degrees higher than most other desert arthropods are able to tolerate. They also conserve water more efficiently than any other arthropod. Some scorpions can live indefinitely without drinking water since water intake can be assured solely by food. (Polis and Sissom, 1990).

1.1.3 Phylogeny and Molecular Studies

Phylogeny is the study of how organisms are related within an evolutionary framework. Scorpions probably constitute a monophyletic taxon (Brownell and Polis, 2001).

Relationships are inferred from a variety of sources, for example, morphological characters, DNA sequences and even behavioral data. Today, DNA sequence data is the most important source of information in the construction of phylogenies. The discovery of the polymerase chain reaction (PCR), the development of automated sequencing and the sophistication of computational power (Caterino et al., 2000) and bioinformatic software greatly potentiated phylogenetic studies in evolutionary biology. Mitochondrial DNA as a molecular marker (Zhang and Hewitt, 1996) remains the most common source of sequence data for studies of mid-to late Cenozoic-age divergences (Sunnucks, 2000).

Mitochondrial DNA is the standard molecular marker in phylogeographic studies, being maternally inherited and usually without recombination and selection (Piganeau et al., 2004). This means that the variants present are not intermixed and so, the mtDNA characters represent the presumed historical sequence of mutation events accompanying the differentiation of maternal lines (Avise et al., 1987; Ballard and Rand, 2005; Ballard and Whitlock, 2004). The mitochondrial is present in all Eukaryotes (there are punctual exceptions) and presents a higher number of equal copies, making this marker easy to assay (Avise et al., 1987; Ballard and Rand, 2005; Ballard and Whitlock, 2004; Kocher et al., 1989). There has to be some caution, however, using mtDNA for phylogenetic analyses. Cases where non-independent replication occurs have been reported (Simon et al., 1994) and this phenomenon was described in scorpions (Gantenbein et al., 2005). Nonetheless, the majority of scorpion

molecular phylogenetic studies choose this marker because there is a comprehensive mtDNA database available and, therefore, a basis for comparative analyses through public databases of DNA sequences. Further, there are no primers for nuclear DNA markers widely used in the Scorpiones order.

1.1.4 Venom and the Buthidae family

All scorpions are capable of producing venom, a complex mixture of more than 200 identified toxins. The degree of toxicity is greatly variable among species. Venom delivery is related with defense against predation and prey incapacitation. Scorpionism (the severe or lethal poisoning by scorpion venom) depends largely on the distribution and abundance of venomous species relatively to human populations. Incidents have high occurrence in American and Asian countries. However, it is in North Africa, particularly in the Saharan area, where it is a public threat (Chippaux and Goyffon, 2008). For example, in Morocco there is an annual mortality of 0.27 per 100 000 inhabitants, but in Tunisia this number can reach levels up to 6.67 deaths per 100 000 inhabitants each year in certain regions (Bouaziz et al., 2008; Touloun et al., 2012). About 30 species of scorpion are recognized as potentially dangerous for humans (Keegan, 1980). With one exception, species with highly potent, mammal-specific neurotoxins belong to a single family, the Buthidae (Koch, 1837). Further details are given in “Chapter II”

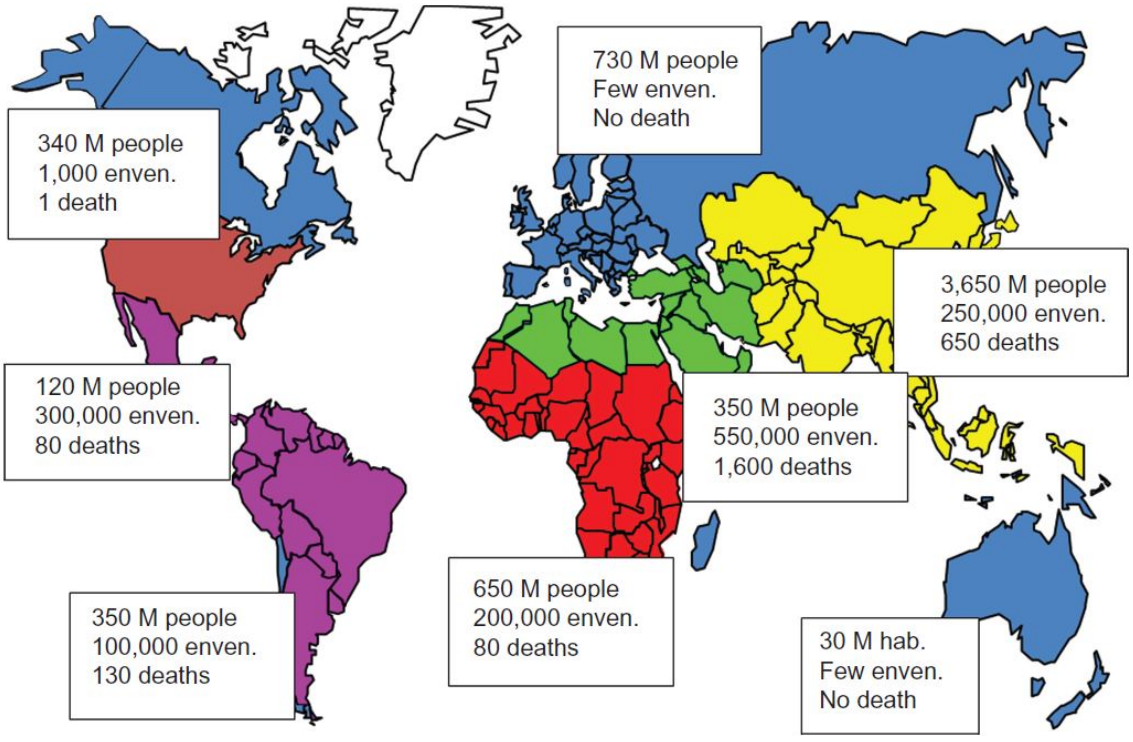


Figure 2: Estimated annual global incidence and mortality following scorpion stings. “Enven” refers to envenomings and “M” refers to million. From Chippaux, 2012)

The Buthidae is the largest scorpion family. This family is known from the fossil record since the Paleocen-Eocene (Baltic amber) and represents one of the basal evolutionary lineages of extant scorpions, so-called orthobothriotaxic Type A (Vachon, 1974). They are ecologically diverse and successful, widely spread across the globe, occupying all six faunal regions (Lourenço, 2000). It contains approximately half of all known extant scorpion species and is divided into 89 genera, most of which are found in the Afrotropical region (Rein, 2012). Until 2005, the Buthidae scorpions were considered to be phylogenetically basal to the other families. Since then, the Pseudochactidae were placed as the most ancient of all extant families (Fet and Soleglad, 2005). While the genus-level diversity in Buthidae is much higher in the Old World compared to the New World, the opposite is observed at the species-level.

The fundamental reason for studying medically relevant scorpions was clearly stated by Simmard and Watt (Polis, 1990): "*we know practically nothing about the natural history or field behavior of any of the deadly species*".

1.3 Objectives

The goal of this thesis was to produce a well supported phylogeny for the genus Androctonus in North-Africa using a combination of DNA markers capable of resolving interspecific relationships. In addition, we also constructed a molecular phylogeny for a broad sampling across all scorpion families. These objectives were accomplished by:

- Testing primers for informative mitochondrial DNA sequence markers for molecular phylogenetics;

- Uncovering levels of Maghrebian *Androctonus* diversity through DNA sequence data from multiple mitochondrial genes;

- Testing the current *Androctonus* taxonomy and biogeographical framework against the resulting molecular dataset;

- Using the same markers to construct a high-level phylogenetic hypothesis of the major groups of scorpions;

CHAPTER II

Preface 1

Deep intraspecific divergences in the medically-relevant fat-tailed scorpions (Androctonus, Scorpiones)

2.1 Preface 1

2.1.1 *Androctonus* scorpions

The Palearctic biogeographic region presents especially rich faunas of arid Buthidae scorpions. Among the most common and widely distributed buthid genera in this region is the *Androctonus* scorpion (Fet et al., 2003). They have a wide geographic range (from Togo to India) and their radiation has been associated with the aridification of the Paleartic deserts (Fet, 1994).

Androctonus scorpions are commonly known as the fat-tail scorpions. They are one of the most common and widely distributed buthid genera from the Paleartic region (together with *Buthus* Leach, 1815, *Hottentotta* Birula, 1908 and *Mesobuthus* Vachon, 1950). The common name is attributed due to the thickness and strength of their metasoma, the "tail", (Figure 3). The name *Androctonus* is derived from a Greek word which roughly translates into "man-killer", a reminder of its highly toxic venom. These scorpions occur in semi-arid and arid regions of desert areas but they have also been found in anthropological disturbed landscapes (Stockmann and Ythier, 2010). They are moderately sized scorpions with adults measuring up to ten centimeters, although larger specimens are known. Preferred habitats include: stony soils, cactus hedges, arid mountainous regions and high plateaus. They can also be found on steep slopes of sand dunes but they avoid humid coastal areas (Gaban, 1997). *Androctonus* usually dig burrows and hide under stones or in natural crevices. They can live four to five years in captivity, depending on the temperature and feeding regime. In terms of toxicity it is one of the most venomous scorpions. *A. australis* LD_{50} values can be as low as 0.32 mg/kg (Goyffon et al., 1982). Notwithstanding, *Androctonus* is popular among exotic animal traders particularly *A. australis* and *A. amoreuxi* which are commonly available. Both the occurrence in the vicinity of human populations and the population densities they reach make *Androctonus* a particularly dangerous genus (Chippaux and Goyffon, 2008).

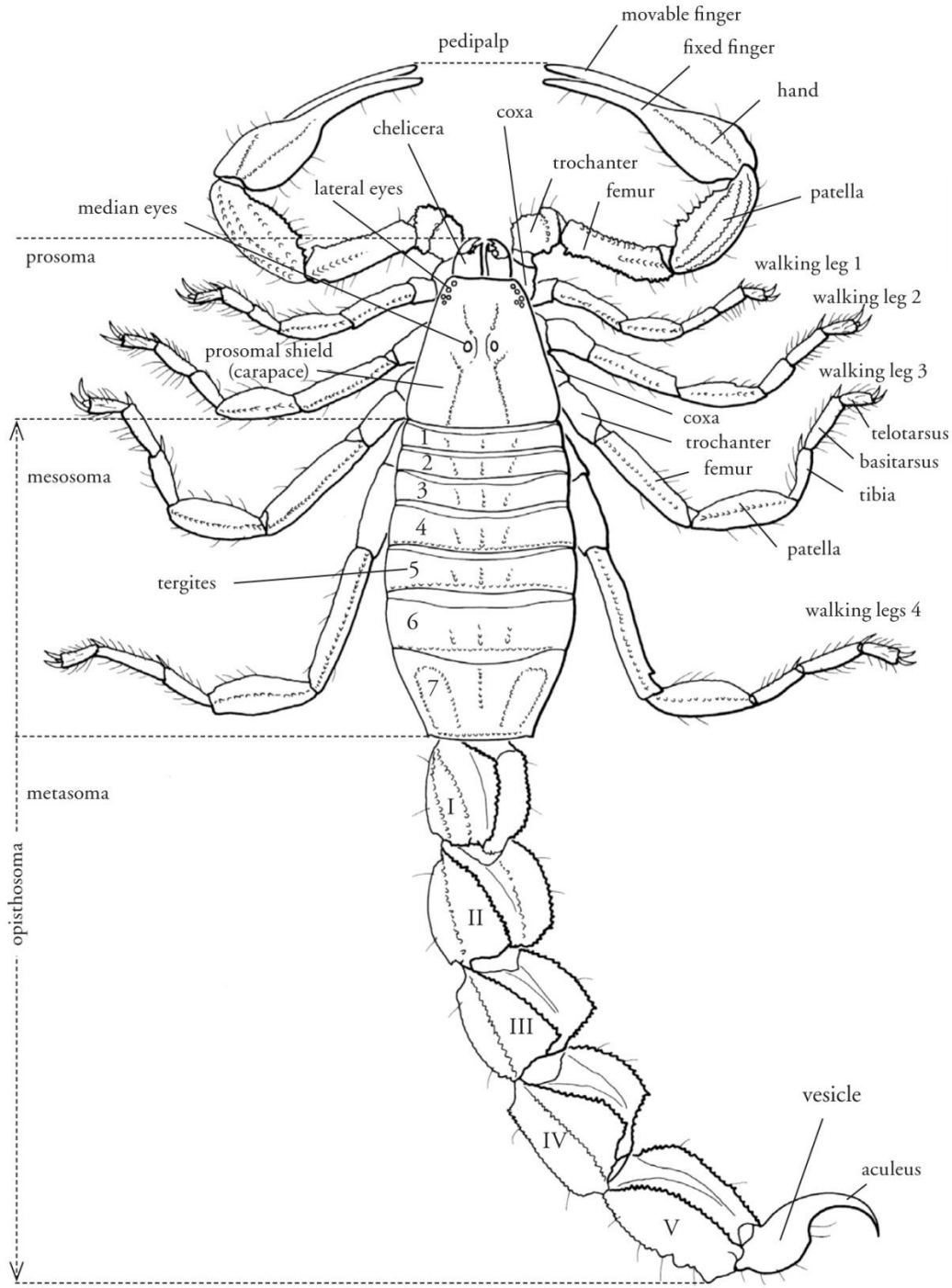


Figure 3: Dorsal view of *Androctonus australis*. The body is divided in two zones, the prosoma, containing the pedipalps, and the ophistosoma. The ophistosoma is subdivided in the mesosoma (abdominal region) and the metasoma bearing the venomous sting. Relevant structures and body segments are listed in the figure (taken from Stockmann and Ythier, 2010).

2.1.2 *Androctonus* taxonomy

The taxonomy of particular scorpion groups is somewhat controversial. *Buthus* scorpions are a clear example, in which more than 15 species have been described in the last decade based solely on morphological characters (*e.g.,* Qi and Lourenço, 2007; Lourenço and Duhem, 2009; Lourenço et al., 2010; Yağmur et al., 2011). Trying to identify them is, for most authors, a daunting task due to conflicting keys and overlapping character ranges. The *Androctonus* genus does not qualify to be considered as complex as the genera *Buthus* or *Tityus*. Nonetheless, *Androctonus* taxonomy underwent repeated taxonomic changes in recent years.

Androctonus scorpions were already known to science since Linnaeus as *Scorpio australis* Linnaeus, 1758 [= *Androctonus australis* (Linnaeus, 1758)], and this remains the type species. Olivier (1807) and Audouin (1826) also described two species under the genus *Scorpio* Linnaeus, 1758. The genus *Androctonus*, as it is recognized nowadays, was later described by Ehrenberg (Ehrenberg and Hemprich, 1828) with two sub-genera *Prionurus* Ehrenberg and *Leiurus* Ehrenberg. The 19th and early 20th centuries were a rich period in *Androctonus* taxonomy: five species and five subspecies attributed to *Androctonus* were described: Olivier (1807), Audoin, (1826), Koch (1839), Pocock (1897, 1900, 1902), (Pallary, 1924) and Caporiacco, 1932. These authors however abandoned the name *Androctonus* and rather used the name *Pionurus* either as a sub-genus of the "catch-all" genus *Buthus* or as a genus itself. With one exception, all described taxa were only later assigned to the genus *Androctonus*. It was only in 1948 that the first of several exhaustive works on North African scorpiofauna were produced by Vachon. As a signature legacy throughout his lifework, Vachon standardized *Androctonus* taxonomy and turned it into a coherent genus (Vachon, 1952, 1949). Following Kraepelin's scheme of diagnostic characters (Kraepelin, 1903) rather than Birula's (Birula, 1910), Vachon's innovative work provided a list of nine *Androctonus* taxa from North Africa, including subspecies. Six of those were described previously though scattered between genera *Buthus* and *Prionurus*; three others were newly described by himself (Table 1). As an influential scorpiologist, Vachon pioneered modern scorpion taxonomy and, as a sign of that, all species he described (with one exception) are still considered valid *Androctonus* taxa today. Later, he would also revise Mauritanian (Vachon, 1953) and Afghan *Androctonus* scorpions (Vachon, 1958). Advances in the second half of the 20th century were more directed towards biochemical taxonomy. Goyffon and Lamy produced important works not only about the morphological differentiation among Tunisian *A. australis* subspecies but also about their biochemical differences (Goyffon and Lamy, 1973; Lamy et al., 1974). This

period was rich on the biochemical and molecular understanding of some of the scorpions' structures and venom. *A. australis* was the main target of these studies but other species were used as well. Few papers on North African *Androctonus* taxonomy were produced, but it is worth noting Fet's contribution with one subspecies, *A. amoreuxi levyi* (Fet, 1997). The most important work in this period would come in the form of a book. Victor Fet and associates compiled and reviewed almost 250 years of scorpion taxonomy. *Catalog of the Scorpions of the World* became one of the most important tools for modern day research in this field (Fet et al., 2000). In it, eight species and 14 subspecies were recognized for *Androctonus*. Few changes were made over Goyffon, Lamy and associates' descriptions but also in Asian species (Table 1). After a halt of almost 60 years since the last major taxonomical revision, Lourenço made a taxonomical review of *Androctonus* (Lourenço, 2005). Important changes included: *A. australis* and *A. mauritanicus* were no longer considered to have subspecies; *A. crassicauda gonneti* was raised to the status of full species; *A. aeneas* was placed in the synonymy of *A. bicolor*; *A. liouvillei* was reconsidered as a valid species (Table 1). Lourenço's contribution did not end with this survey. As a scorpiologist with more than 360 scorpion taxa described, he introduced four more *Androctonus* species and revalidated another one. Today, the genus *Androctonus* features 19 species of which almost two thirds occur in North Africa (see Table 1).

Table 1 *Androctonus* taxonomy – Taxonomical descriptions compiled in three scorpiologists works. Central African and Asian taxa are omitted. (*A. aeneas* = *A. bicolor*).

	Taxon	Author	Year		Taxon	Author	Year
Vachon, 1948, 1952	*A. australis*	Linnaeus	1758	**Fet *et al*., 2000**	*A. australis*	Linnaeus	1758
	A. crassicauda	Olivier	1807		*A. crassicauda*	Olivier	1807
	A. amoreuxi	Audouin	1826		*A. amoreuxi*	Audouin	1826
	A. aeneas	Koch	1839		*A. bicolor*	Ehrenberg	1828
	A. mauritanicus	Pocock	1902		*A. mauritanicus*	Pocock	1902
	A. hoggarensis	Pallary	1929		*A. hoggarensis*	Pallary	1929
	A. sergenti	Vachon	1948		*A. sergenti*	Vachon	1948
Lourenço, 2005	*A. australis*	Linnaeus	1758	**Current taxonomy**	*A. australis*	Linnaeus	1758
	A. crassicauda	Olivier	1807		*A. crassicauda*	Olivier	1807
	A. amoreuxi	Audouin	1826		*A. amoreuxi*	Audouin	1826
	A. bicolor	Ehrenberg	1828		*A. bicolor*	Ehrenberg	1828
	A. mauritanicus	Pocock	1902		*A. mauritanicus*	Pocock	1902
	A. liouvillei	Pallary	1924		*A. liouvillei*	Pallary	1924
	A. hoggarensis	Pallary	1929		*A. hoggarensis*	Pallary	1929
	A. gonneti	Vachon	1948		*A. gonneti*	Vachon	1948
	A. sergenti	Vachon	1948		*A. sergenti*	Vachon	1948
					A. aleksandrplotkini	Lourenço & Qi	2007
					A. maroccanus	Lourenço, Yhtier & Leguin	2009

2.1.3 Why study *Androctonus* scorpions?

The first molecular phylogeny of the family Buthidae (Fet *et al*., 2003) included a sequence of *A. amoreuxi*. This work was the first and, so far, the only DNA phylogeny between buthid genera. With a weak support, *Androctonus* is placed more closely together with *Leiurus* Ehrenberg, 1828 rather than with *Buthus* (Figure 4). This was a surprising result because, apart from historically being confused with one another morphologically, *Androctonus* and *Buthus* scorpions share the same number and arrangement of pedipalp manus granules (3+1 terminal). On the other hand, the proposed clade unites the most venomous species pair of scorpions: *Androctonus australis* and *Leiurus quinquestriatus* (Ehrenberg, 1828). The importance around the relationship between toxic scorpions directly relates to the emergence of such potent venoms.

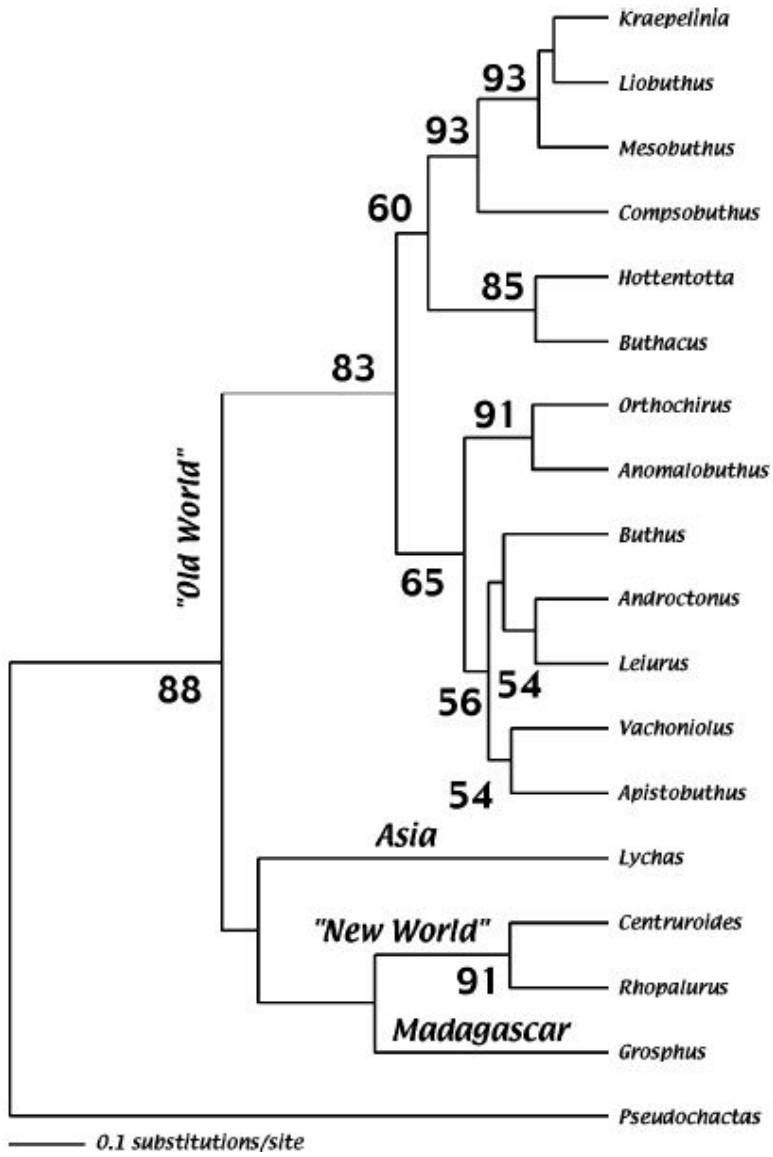


Figure 4: Maximum Likelihood tree of Old World and New World Buthidae of the 16S mtDNA region. From Fet et al., 2003.

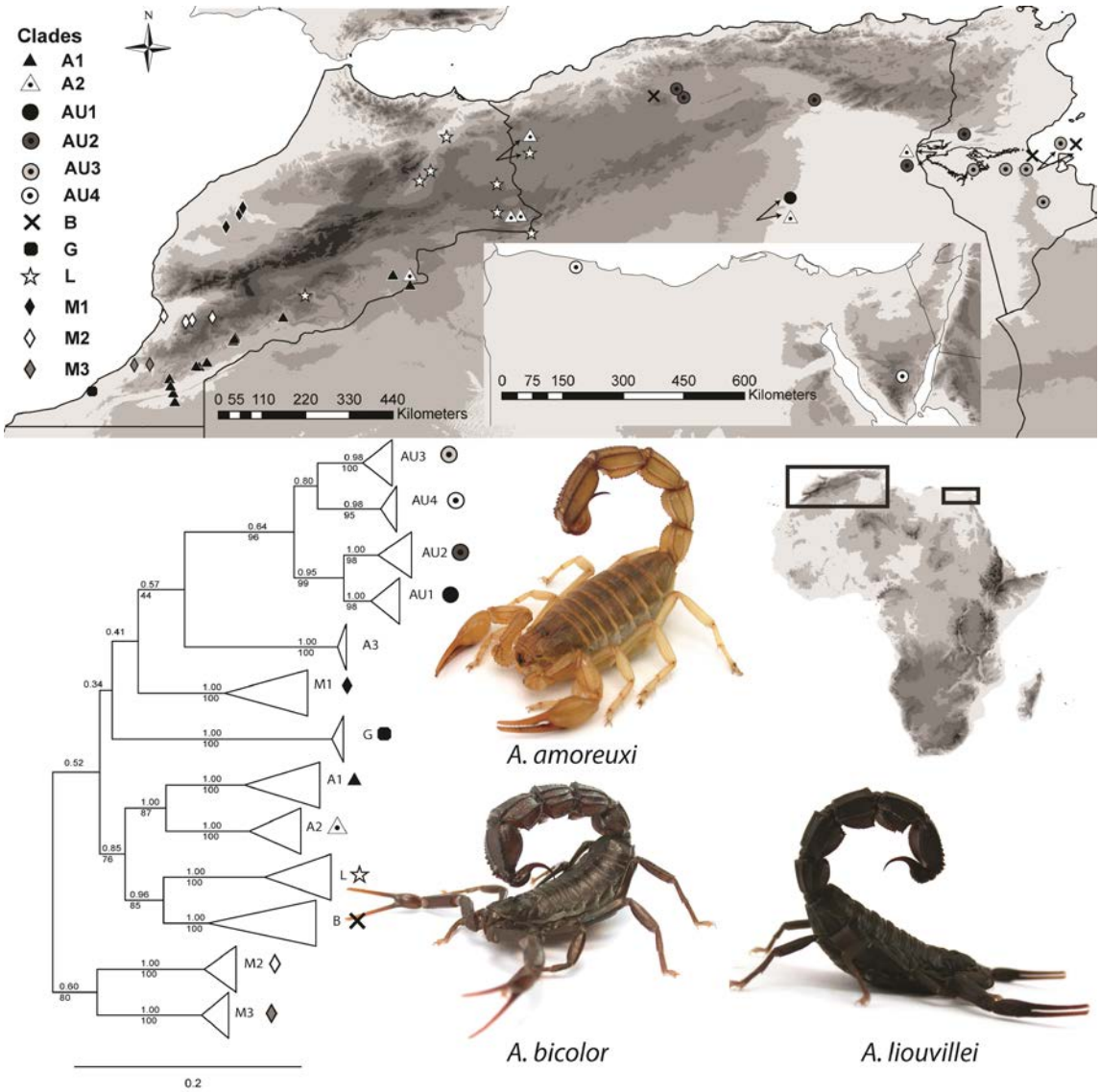
The study of venom toxins provides insights on scorpion evolution as well. For instance, New World buthids have separate mammal and insect-specific neurotoxins for Na^+ channels but Old World buthids have non-specific toxins that act both on mammals and insects. This independent evolution of venoms probably emerged after the Laurasia *versus* Gondwana split and is a sign of the selective pressures during the aridification of the Paleartic in the Tertiary period (Fet et al., 2003). This period witnessed a rapid radiation of small burrowing mammals in arid landscapes. Rodents would be a direct competitor for territory in the subsoil but also important nocturnal predators as is the case today (McCormick and Polis, 1990). This could explain why specific mammal targeted toxins used in defense are present in burrow-living scorpions rather than vegetation-inhabiting New World buthids. There are, however, some

exceptions of scorpion toxins that follow a different path on sodium channels: they act both in alpha and beta sites of these proteins' domains. They were first isolated in *A. australis* starting a discussion that mammal-targeted buthid toxins "*originated from North Africa*" (Loret and Hammock, 2001). Although an interesting statement it is not testable given our current knowledge:

- The Asian range of *A. australis* is still debated. Lourenço (2005) defined Egypt as the eastern limit of distribution raising the middle eastern *A. australis baluchicus* to species level;
- The center of origin is unknown. There is no wide biogeographical study of *Androctonus* distribution, diversity and relationship among their species;
- The toxin in question, AahIT4, or related toxins have not been tested in an evolutionary framework mostly because these experiments are nearly impossible to replicate. This happens because toxicological literature provides poor taxonomic identifications. *A. australis* is often oversimplified as the "*Sahara scorpion*" and sampling has equally poor georeferencing (sometimes non-existent).

As highlighted by Victor Fet and coworkers (2003), technical difficulties such as the above mentioned lead to "*unjustified and superficial conclusions about evolution of both species and toxins*". A molecular study of *Androctonus* scorpions would serve as the starting point to overcome some obstacles by providing quantitative criteria to delimit taxa and species distribution. In conjugation with the available buthid phylogeny, the exceptional properties of *A. australis* can be tested as an ancestral or synapomorphic character, a key feature to understand scorpion evolution. More recently, similar compounds have been identified in *Centruroides suffussus suffusus* (Espino-Solis et al., 2011).

2.2 Deep intraspecific divergences in the medically-relevant fat-tailed scorpions (*Androctonus*, Scorpiones)



(Graphical abstract)

Deep intraspecific divergences in the medically-relevant fat-tailed scorpions (*Androctonus*, Scorpiones)

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Keywords: *Androctonus*; Scorpions; Phylogeny; Biogeography; Cryptic Diversity

Abstract

North Africa is the major hotspot of scorpion sting incidents in the world, and *Androctonus* scorpions, commonly known as fat-tailed scorpions, are among the most medically relevant scorpions in North Africa. Since venom composition within species is known to vary regionally, the improvement of therapeutic management depends on a correct assessment of the existing regional specific and sub-specific variation. The genus *Androctonus* contains 19 species distributed from Togo and Mauritania in the west, North Africa, through the Middle East and to as far east as India. With ten species, a substantial amount of this genus' diversity occurs in North Africa, although other regions remain understudied. In this study, we assessed the phylogeographical patterns in six species of *Androctonus* scorpions from North Africa using mitochondrial DNA markers. We sequenced COI, 12S, 16S and ND1 genes from 110 individuals. Despite lacking basal resolution in the tree we found taxonomical and geographically coherent clades. We discovered deep intraspecific variation in the widespread *A. amoreuxi* and *A. australis*, which consisted of several well supported clades. Genetic distances between some of these clades are as high as those found between species. North African *A. australis* have a deep split in Tunisia around the *Chott el-Djerid* salt-lake. A surprising split between *A. amoreuxi* scorpions was found in Morocco. We also found deep divergences in *A. mauritanicus,* corresponding to areas attributed to invalidated subspecies. In addition we uncovered a clade of specimens from coastal south Morocco, which could not be ascribed to any know species using morphological characters. Based on these findings we recommend a reassessment of venom potency and anti-venom efficacy between these deep intraspecific divergent clades.

1. Introduction

Worldwide, 1.2 million people are stung by scorpions every year. Scorpionism, defined as the severe to lethal incident as a consequence of a scorpion sting (Lourenço and Cuellar, 1995) may be responsible for 3250 global annual mortalities which are mostly concentrated in a few high-risk areas (Chippaux and Goyffon, 2008). North Africa in particular is considered a high-risk area for scorpionism (Chippaux and Goyffon, 2008), with the genera *Leiurus* and *Androctonus* being the foremost cause of serious envenomation in this area (Goyffon and Guette, 2005; Graham, 2011; Habermehl, 1994). Five *Androctonus* species are considered as dangerous to man, particularly the widespread *A. australis* (Linnaeus, 1758) and *A. mauritanicus* (Pocock, 1902), which are the most dangerous *Androctonus* in the Maghreb region (Goyffon and Guette, 2005). *A. australis* is known for dangerously envenomating humans and possessing a high toxicity (LD_{50} = 0.32 mg/kg; Watt and Simard, 1984). For this reason, *A. australis* was one of the first species of scorpions to have its venom purified for neurotoxin characterization (Miranda et al., 1966). As in snakes (Daltry et al., 1996; Prasad et al., 1999), scorpion venom is known to have considerable intraspecific regional variation in composition (el Ayeb and Rochat, 1985; Newton et al., 2007; Smertenko et al., 2001), and thus a different response to antivenom treatment (Omran and McVean, 2000). Furthermore, other species such as *A. amoreuxi* (Audouin, 1826) may also cause more cases of scorpionism than currently thought (Goyffon et al., 2012). It is therefore important to study the phylogeographical patterns of *Androctonus* over a great part of their distribution as it may have direct applications in therapeutic management.

The scorpion genus *Androctonus* was first described by Ehrenberg in 1828. Vachon (1952) stabilized the genus' taxonomy, transforming it into a morphological and geographical coherent group with seven species known in North Africa. Lourenço (2005) produced an important taxonomical revision of the genus: the subspecies of *A. australis* and *A. mauritanicus* were no longer considered valid, *A. crassicauda gonneti* Vachon, 1948 was raised to the status of species, *A. aeneas* C. L. Koch, 1839 was placed in the synonymy of *A. bicolor* Ehrenberg, 1828 and *A. liouvillei* (Pallary, 1924) was raised to the species level. Since then, two more taxa were described from North Africa, *A. aleksandrplotkini* Lourenço & Qi, 2007 and *A. maroccanus* Lourenço Yhtier & Leguin, 2009, and one was reconsidered as a valid taxon, *A. eburneus* Pallary, 1928.

The genus is distributed from Togo to Morocco in the Atlantic coast of Africa (Lourenço and Qi, 2007; Lourenço, 2008) to the Maghreb countries and Egypt (Vachon, 1952), the Middle East (Levy and Amitai, 1980), reaching across Afghanistan (Vachon, 1958) to India (Tikader and Bastawade, 1983). Some species have been cited from Saudi Arabia, Yemen and Lebanon, but these records require additional confirmation (Fet et al., 2000). In this work, we assess five species of the Maghrebian countries except for Libya, plus Egypt and the Sinai Peninsula. *A. mauritanicus* occurs in Morocco. This country shares a further three species with Algeria, Tunisia and/or Egypt (*A. liouvillei*, *A. australis* and *A. amoreuxi*), while *A. bicolor* occurs in Algeria and Tunisia.

The radiation of buthids has been associated with the aridification of the Palearctic Region (Fet et al., 2003, 1998). Little is known about the biogeography of *Androctonus* however. Recently, molecular tools have been used to assess the phylogeny of some species in Tunisia. Ben Ali et al., (2000), using nuclear DNA ITS regions (ITS-rDNA), found paraphyletic clades in three well accepted taxa (*A. bicolor*, *A. australis* and *A. amoreuxi*). With the same outcome, Ben Othmen et al., (2004), using allozymic differentiation, found little support for the monophyly of *A. australis* and *A. amoreuxi* individually. However, the same authors recovered three well-supported monophyletic lineages using 16S-rDNA, each corresponding to a species, thus demonstrating the usefulness of this gene as a barcoding marker (Ben Othmen et al., 2009). Furthermore, they found a phylogenetic pattern of *A. australis* in Tunisia, where each of its two lineages are distributed to the north or south of the *Chott el-Djerid* salt lake. However their molecular dating makes it unlikely that the salt lake formation has generated a vicariant evolution of Tunisian *A. australis*. In recent years, molecular studies using the mitochondrial COI gene already uncovered considerable cryptic diversity in other scorpion genera in the Maghreb region (Gantenbein and Largiadèr, 2003; Sousa et al., 2012, 2011, 2010).

In this study, we assess the patterns of diversity estimated from COI, 16S and 12S sequence data across the Maghreb region and Egypt. We provide the first molecular phylogeny for *Androctonus* scorpions in Morocco, Algeria and Egypt.

2. Materials and Methods

2.1 **Taxon sampling**

We collected samples in the field in three countries: Morocco, Tunisia and Egypt (figure 1). Algerian samples were donated by Dr. Said Larbes. Additional specimens were purchased through the pet-trade. Sampling points are illustrated in Figure 1 and further details are provided in Table 1. Scorpions were captured with long forceps and preserved in 96% ethanol. To minimize the impact on scorpion populations, non-lethal sampling methods were used when possible, and consisted of removing the distal part of one of the second leg pairs. The scorpion was subsequently placed in the sand, facilitating the clotting process and thus minimizing haemolymph loss. Scorpions can recover partially amputated appendages after a few molts and it is common for scorpions to be active and functional even when missing appendages (pers. obs.). Voucher specimens were collected at selected sites. All specimens are deposited in the collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Vila do Conde, Portugal.

Some key species that could not be sampled in the field, *e.g.* Egyptian *A. bicolor*, were obtained through reputable animal traders. Morphological identification was done in the lab using keys by Vachon, (1952) and Lourenço, (2005). A single specimen of *Opisthacanthus asper* (Peters, 1861) (Scorpiones: Liochelidae) was used as outgroup (details provided in Table 1).

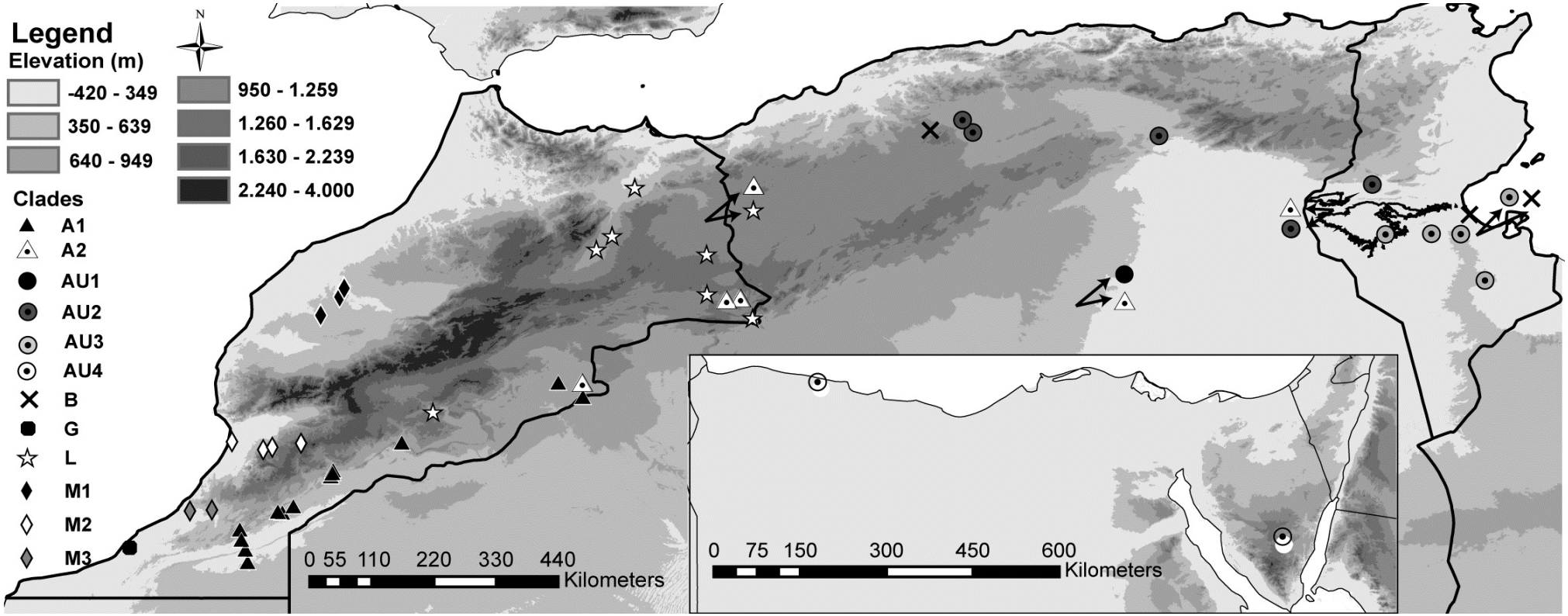

Figure 1: Map representing the sampling locations across North Africa of *Androctonus* scorpions. Insert shows Egyptian samples. Pet trade acquired samples were without locality data, and are not shown. Symbols correspond to the phylogenetic clades (see figure 2).

DNA extraction and PCR conditions

Fresh or preserved leg muscle tissue (or metasoma muscle for smaller specimens) was used for DNA extraction. Dissection occurred, preferentially, from the third leg in order to minimize the loss of important taxonomical characters. Total DNA was extracted using proteinase K digestion (10 mg/ml concentration) followed by a standard salt extraction protocol (Bruford et al., 1992).

Four mitochondrial fragments were amplified: 16S ribosomal RNA (16S rRNA), 12S ribosomal RNA (12S rRNA), Cytochrome C Oxidase subunit 1 (COI) and NADH Dehydrogenase 1 (ND1) (see Table 2). Polymerase chain reactions were performed in a final volume of 25 µL and using 1.0 µL each of 10 pmol primer, 12.5 µL REDTaq ReadyMix PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich, St. Louis, USA), 1.0 µL of the purified DNA and 9.5 µL of water. Difficult PCR reactions were amplified with a Touchdown-PCR approach. Purified PCR templates were sequenced using dye-labelled dideoxy terminator cycle sequencing on an ABI 3730XL at Macrogen Inc. The sequencing primers were the same as those used in the PCRs.

Table 2: List of primers and PCR conditions used for molecular analyses. PCR conditions start with temperature (ºC) of each step followed by the time in seconds in brackets.

Gene	Primer name	Sequence (5' → 3')	Source	PCR conditions
16S rRNA	18-mer (forward)	CGATTTGAACTCAGATCA	*Simon et al, 1994*	94(180), [94(30), 50(45), 72(60)]x35, 72(300), 12(∞)
	20-mer (reverse)	GTGCAAAGGTAGCATAAT	*Gantenbein et al, 2000*	
12S rRNA	12S-F_AvdM	AGAG-TGACGGGCAATATGTG	*van der Meijden et al, 2012*	94(180), [94(30), 52(45), 72(60)]x35, 72(300), 12(∞)
	12S-R_AvdM	CAGCGGCTGCGGTTATAC		
COI	LCO1490 (forward)	GGTCAACAAATCATCATAAAGATATTGG	*Folmer et al. 1994*	94(180), [94(30), 48(45), 72(60)]x35, 72(300), 12(∞)
	HCO219 (reverse)	TAAACTTCAGGGTGACCAAAAAATCA		
	COI_avdm_F	WTYCTACIAATCAYAARGATATTGG	*van der Meijden et al, 2012*	94(180), [94(30), 49(45), 72(60)]x35, 72(300), 12(∞)
	COI_avdm_R	TAMACYTCIGGGTGWCCAAAAAAYCA		
ND1	ND1-LR-N-12945 (forward)	CGACCTCGATGTTGAATTAA	*Hedin, 1997*	94(180), [94(30), 47(45), 72(60)]x35, 72(300), 12(∞)
	ND1-N1-J-12261 (reverse)	TCGTAAGAAATTATTTGAGC		

2.3 Data analysis

Chromatographs were checked manually for sequencing errors using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, USA). Sequences were edited using MEGA 5 (Tamura et al., 2011) and aligned using default settings of MUSCLE (Edgar, 2004) for non-coding sequences and ClustalW (Larkin et al., 2007) for COI and ND1.

Phylogeny reconstruction was performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. A homogeneity partition test executed in Paup* 4.0 rejected homogeneity of the different genes. We therefore carried out both a portioned and a combined analysis. The best fitting models of sequence evolution were determined by the AIC criterion in JModeltest 2.1.2 (Darriba et al., 2012) both for the separate genes and the combined dataset. ML tree searches were performed using PhyML, version 3.0 (Guindon et al., 2010). Bootstrap branch support values were calculated with 1000 replicates. The BI analysis was conducted with MrBayes 3.2.2, with 5.000.000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 12.500 trees). Mean genetic distances between and within clades were calculated with MEGA5 based on COI sequences. Variance was estimated with 4000 bootstrap replicates, with uncorrected p-distances (Table 3). GenBank accession numbers are given in Table 1.

In addition we downloaded all 16S rDNA sequences in Ben Othmen et al., (2009) from GenBank and we combined these with our 16S dataset. Phylogeny reconstruction of this extended dataset was performed with ML (tree not shown).

3. Results

3.1 Sequence data

We sequenced 110 *Androctonus* specimens and one outgroup (O. *asper*) (Table 1). The combined dataset consists of an alignment of 1457 basepairs (bp) (606 bp of COI, 374 bp of 16S rRNA and 477 bp of 12S rRNA). The dataset included 777 variable sites (53.3 % of the total nucleotide positions), 680 bp were constant (46.7 %) and 511 positions were parsimony informative (35.1%). Due to difficulties in amplifying ND1 only 41 sequences were available, and thus this gene was not included in the combined analysis.

3.2 Overall phylogeny

Four of the six species were recovered as monophyletic (Figure 2). We identified 13 clades that correspond to biogeographical and/or taxonomical units. Unfortunately, most of the deeper branches present low values of bootstrap support (ML) and posterior probability (BI). Consequently, the relationships between species could not always be resolved. ML and BI trees using a dataset that included ND1 sequences (not shown) did not differ in the topology of well supported clades. Trees derived from the individual genes did not contradict the result from the concatenated dataset.

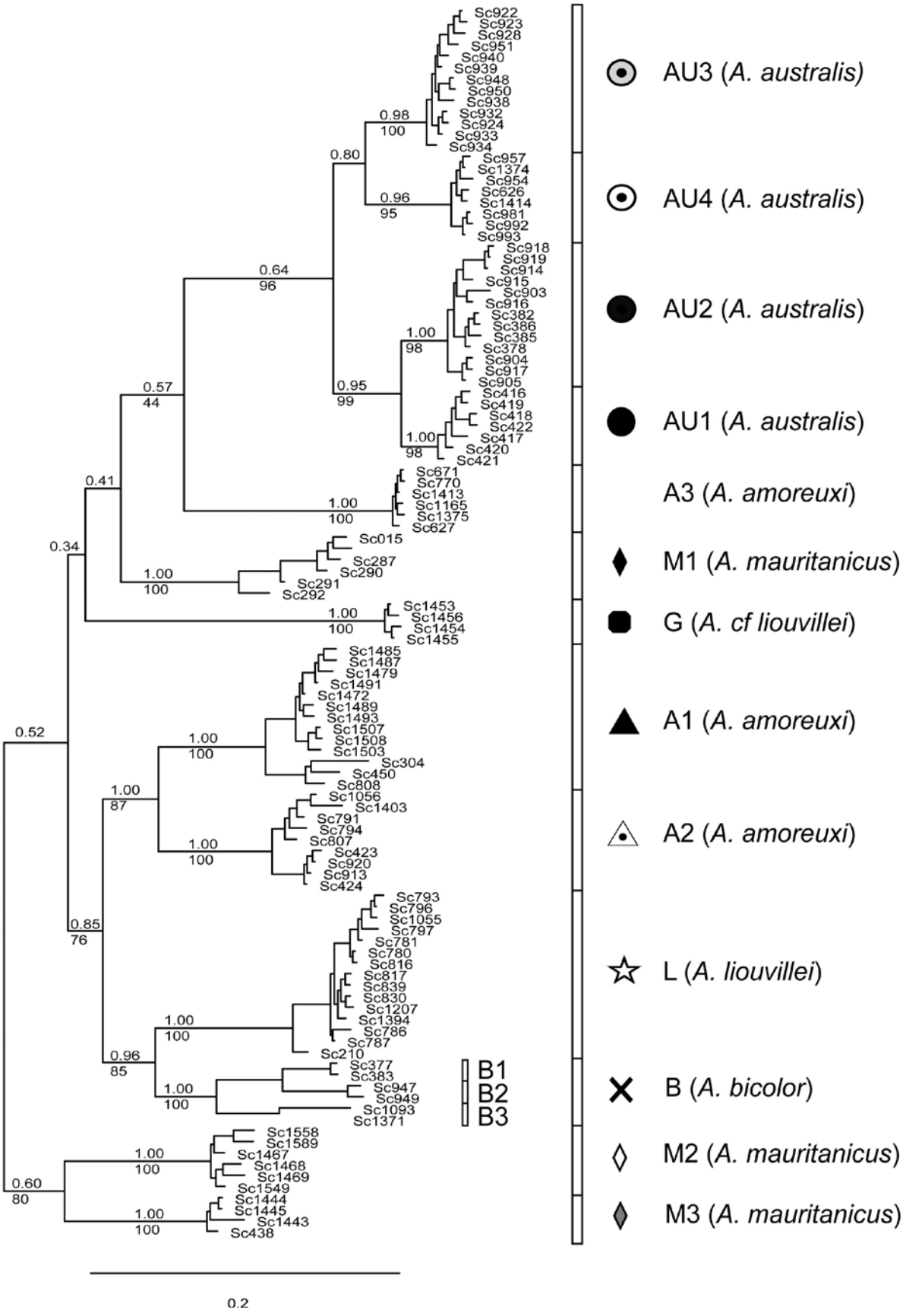

Figure 2: Estimate of phylogenetic relationships of *Androctonus* (outgroup not shown). Posterior probabilities values and bootstrap support values are shown above and below nodes respectively. These samples are marked with the same colors and shapes in all figures.

3.3 Species-level clades

Androctonus amoreuxi, is divided into three clades, two of them containing specimens from Morocco (A1 and A2), Tunisia and Algeria (A2) and one from Egypt (A3). Clades A1 and A2 are placed as sister groups with a high support (BI = 1.00; ML = 87) and the A3 clade has no well-supported affinities but may be sister taxon to *A. australis*. The western part of the range, from Morocco to Tunisia, is divided into the two clades; a northern (A2) and a southern clade (A1); the Southern Moroccan *amoreuxi* (A1) clade extends South, from the regions of Assa and Zag in the South, to the southern area of the Anti-Atlas Mountains' and North towards Taouz, in the Erfoud area, close to the Morocco-Algerian border, but also in the area of Midelt, in the Middle-Atlas Mountains (figure 1). The Northwest African *amoreuxi* (A2) clade groups specimens from three countries: in Morocco it is distributed in the Erfoud area and also to the areas surrounding Bouarfa and Ain-Beni-Mathar (High Plateau in Northeast Morocco); In Algeria it is represented in one location, Ghardaia (between the Great Ergs); in Tunisia it is represented in one location, close to Tozeur (between *Chott el-Gharsa* and *Chott el-Djerid* salt lakes). The third clade, A3 groups specimens that were acquired via the pet-trade from Egypt, none of which are georeferenced. Pairwise genetic distances between these clades are in a range between 7.5% and 9.2% (Table 3). Downloaded 16S sequences of *A. amoreuxi* are split between clades A1 and A2 with Ben Othmen et al., (2009)'s sequence (andr05) placed in the A2 clade whereas the sequence of (Fet et al., 2003) is placed within our A1 clade (tree not shown).

The L clade (*liouvillei* clade) unites all Moroccan *A. liouvillei* scorpions. A total of 15 specimens were collected, ranging from the High-Atlas Mountains (Agdz), to the Northeastern range of the Middle-Atlas (Outat El Haj) and to the High Plateau along the Algerian borderland (e.g. Figuig). Average genetic distance within the clade is of 0.8%. It appears to be sister taxon to *A. bicolor*.

A. mauritanicus, is presented in three clades; M2 and M3 are sister clades (BI=0.60; ML=80) whereas M1 was unrelated to these. Specimens from the M1 clade are located in the northern range of the Marrakech-Tensif-El Haouz administrative region. The specimens from the M2 clade occur in a horizontal stretch from the peaks of the Anti-Atlas (close to Ighern) along the Souss valley with the westernmost sample being 6 km from the seacoast. The M3 clade is comprised of specimens from two sampling locations, both from the Guelmim area in the southwest slope of the Anti-Atlas mountains (see figure 1). Genetic distances between clades range from 9.0% to 10.5% while the highest genetic distance within these clades, in clade M2, is 2.3% (Table 3).

A group of four dark-colored *Androctonus,* which morphologically resemble *A. liouvillei* most closely, are designated here as *A.* cf. *liouvillei* (see section 4). These specimens (clade G) were found in one sampling point in the southern limit of the Anti-Atlas Mountains near the mouth of the Oued Draa.

Androctonus australis forms a monophyletic unit, divided into four clades. The AU3 (east Tunisian *australis*) and AU4 (Egyptian *australis*) clades are sister groups in the Bayesian tree (BI = 0.80), which is not the case in the ML analysis. Clades AU1 (Algerian *australis*) and AU2 (west Tunisian *australis*) are sister groups in both trees (BI=0.95; ML=99). Starting from the East, the AU4 clade is exclusive to Egypt and is distributed in at least three areas: Mersa Matruh, the West Mediterranean coast of Egypt and north from Mount Sinai. Some of the specimens present in this clade are not geographically referenced however, as they were obtained through the pet-trade. The AU3 clade (East Tunisian *australis*) is distributed solely in Tunisia: specimens range as far west as Kebili (Southeast of the *Chott el-Djerid* salt lake), north to the *Chot el-Fejaj* salt lake and as far south as the Tataouine desert region (Figure 1). The AU2 clade groups specimens from two countries: Tunisia and Algeria. In Tunisia this clade is distributed in an area compressed between Gafsa (in the east), *Chott el-Djerid* salt lake (in the South) and *Chot el-Gharsa* salt lake (in the west). In Algeria this clade is spread in two regions: in M'Doukal, a region enclosed between the Ouled-Nail (in the southwest), the Tell Atlas (in the North) and the Aurès mountains (in the East); and near Hassi Fedoul, at the foot of the Tell Atlas, the salt water lakes (in the west) and the Ouled-Nail mountains (in the east). The AU1 clade is comprised of specimens from a single location, Ghardaia, located between the Great Ergs of Algeria (Figure 1). Pairwise genetic distances between these clades range from 3.9% to 8.0%. The AU1 and AU4 clades present the lowest values of within group genetic distance (0.4%). Among *A. australis,* the highest within group distance was found in clade AU2 (mean p-distance = 1.2%). Our sampling confirms former suspicions on this species' occurrence in the Sinai (Levy and Amitai, 1980). The extended 16S dataset shows that Ben Othmen et al., (2009)'s "northern and southern lineage" sequences are placed throughout the AU2 and AU3 clades respectively (tree not shown).

The B clade (*bicolor* Clade) unites all *A. bicolor* specimens. Notably, this clade has longer branches within it than any of the other clades in this study. For this reason we divided this clade into three subclades (B1, B2 and B3) and quantified the genetic distances between them. *A. bicolor* is present in Hassi Fedoul (Tell Atlas, Algeria), near Gabès (central Tunisia) (Figure 1) and Egypt (no GPS point available). Genetic distances between these subclades range from 4.2% to 7.9%. These subclades were too small for mean p-distance within clades to be calculated.

Table 3 *Genetic distances between and within groups*. Numbers below the diagonal are mean p-distances calculated between clades whereas the corresponding variances, based on 4000 bootstrap replicates, are shown above the diagonal. Mean p-distances within clades are shown to the right.

	Distance between clades															Within Clades		
A1		0,010	0,011	0,011	0,011	0,011	0,011	0,011	0,010	0,011	0,010	0,010	0,011	0,011	0,012	0,016	0,002	**A1**
A2	0,075		0,011	0,012	0,011	0,012	0,012	0,010	0,011	0,011	0,010	0,011	0,011	0,011	0,011	0,013	0,003	**A2**
A3	0,092	0,089		0,012	0,011	0,011	0,011	0,012	0,012	0,011	0,011	0,011	0,012	0,011	0,013	0,003	0,001	**A3**
AU1	0,090	0,102	0,090		0,007	0,009	0,011	0,012	0,011	0,012	0,012	0,011	0,012	0,012	0,012	0,004	0,002	**AU1**
AU2	0,087	0,102	0,093	0,039		0,010	0,010	0,012	0,011	0,012	0,011	0,011	0,011	0,011	0,012	0,012	0,003	**AU2**
AU3	0,090	0,098	0,097	0,061	0,069		0,009	0,012	0,011	0,011	0,012	0,010	0,011	0,011	0,012	0,009	0,002	**AU3**
AU4	0,094	0,093	0,089	0,080	0,080	0,056		0,013	0,011	0,012	0,012	0,011	0,012	0,012	0,012	0,004	0,001	**AU4**
B1	0,088	0,086	0,096	0,093	0,090	0,097	0,108		0,008	0,011	0,011	0,012	0,011	0,012	0,012	n/c	n/c	**B1**
B2	0,077	0,082	0,100	0,093	0,087	0,087	0,096	0,042		0,010	0,011	0,011	0,011	0,011	0,013	0,010	0,004	**B2**
B3	0,094	0,100	0,099	0,106	0,099	0,098	0,107	0,079	0,074		0,011	0,010	0,011	0,011	0,012	n/c	n/c	**B3**
L	0,087	0,081	0,096	0,099	0,099	0,108	0,108	0,087	0,085	0,083		0,011	0,011	0,011	0,011	0,008	0,002	**L**
M1	0,084	0,098	0,095	0,087	0,087	0,081	0,096	0,085	0,080	0,090	0,096		0,011	0,011	0,012	0,012	0,003	**M1**
M2	0,110	0,103	0,115	0,109	0,108	0,099	0,116	0,096	0,099	0,094	0,099	0,105		0,010	0,011	0,023	0,004	**M2**
M3	0,103	0,104	0,097	0,107	0,102	0,100	0,107	0,109	0,104	0,095	0,098	0,093	0,090		0,011	0,014	0,003	**M3**
G	0,101	0,087	0,111	0,100	0,104	0,096	0,102	0,109	0,109	0,106	0,085	0,111	0,104	0,088		0,004	0,002	**G**
	A1	**A2**	**A3**	**AU1**	**AU2**	**AU3**	**AU4**	**B1**	**B2**	**B3**	**L**	**M1**	**M2**	**M3**	**G**	**p-distance**	**St. err.**	

4. Discussion

Genetic methods, using one or very few mitochondrial genes, have proven very successful in uncovering cryptic diversity in North African scorpions (Froufe et al., 2008; Sousa et al., 2012, 2011). Despite using three genes with a high number of informative sites, the dataset did not provide sufficient resolution below the species level. The relationships among these species therefore could not be resolved. Species-level clades, however, were well resolved and received high support

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4.1 *Androctonus australis*

There is a deep split between clades AU1 and AU2 on one side, and clades AU3 and AU4 on the other. The two clades from the same species in Tunisia (clades AU2 and AU3) are therefore more closely related to clades distributed in countries as far east as Egypt (AU4) and as far west as Algeria (AU1) than to each other. The genetic distance also reflects a higher distance between them than between the pairs AU1-AU2 and AU3-AU4. Geographical factors might explain the intraspecific variation observed. Tunisia has a heterogeneous landscape, with a mountainous North and low-lying deserts in the South, separated by salt lakes. There is a large salt lake between

the regions where clades AU2 and AU3 are situated. Salt lakes as big as *Chott el-Djerid* might act as physical barriers for scorpion dispersion, as suggested by Ben Othmen et al. (2009). Although their work dealt only with Tunisian *Androctonus*, had a smaller sampling and used only 16S rRNA data, they also found a deep divergence between the north-western and south-eastern Tunisian *A. australis* the same as the one found between the clades AU2 and AU3. Their "northern" and "southern lineages" are genetically and geographically congruent with the AU2 and AU3 clades respectively. They explored the possibility of the lake's formation causing the vicariant event. However the dates for the lake's formation do not coincide with their divergence time estimation. The geological evidence on the date of formation of *Chott el-Djerid* salt lake is ambiguous; it is dated to be 65 Ma old if it is considered the remains of an Eocene inland sea (Levy and Amitai, 1980), or it is dated between 90 ka – 150 ka if it is considered a continental lake formed by tectonic and climate changes (Ben Othmen et al., 2009). Ben Othmen et al. (2009) calculated the date for the divergence of the Tunisian lineages to be 3.43 - 9.90 Ma and thus, unlikely an effect of *Chott el-Djerid*'s formation in either paleogeographic scenario. However, their data divergence time estimate was not based on calibration points, but rather on a constant mutation rate, and should be regarded with caution. Alternatively, the species may have become split into two major lineages on either side of the holocene humid corridor (Drake et al., 2011), which divided the Sahara in a roughly south-north direction. The recent desertification of this humid corridor would have allowed the two diverged lineages to come into secondary contact around *Chott el-Djerid*. Unpublished data shows *Chott el-Djerid* also acts as a geographical barrier for *Buthus* scorpions (Pedroso *et al.*, in prep.). Further studies of this contact zone towards the south, in conjunction with proper molecular divergence time estimates, could be helpful in understanding the history of the holocene humid corridor, and the recent desertification process of the Sahara desert.

A. a*ustralis* is one of the most widespread scorpions in North Africa and one of the leading causes of scorpionism in North Africa (Chippaux and Goyffon, 2008). Since venom composition and efficacy is known to exhibit regional variation both in snakes (Daltry et al., 1996; Prasad et al., 1999) and scorpions (Newton et al., 2007; Omran and McVean, 2000), these two attributes may also differ between the clades we identified in this study, and should be investigated separately.

4.2 *Androctonus mauritanicus*

A. mauritanicus is, together with *A. australis,* among the most dangerous scorpions in the Maghreb (Chippaux and Goyffon, 2008). Between 1996 and 2006 in Morocco, 53% of deaths that resulted from sting cases come from incidents with *A. mauritanicus* (Touloun et al., 2012). Although these scorpions are found in median altitudes in some of the highest mountains of Morocco they do not seem to be restricted to the mountains, because specimens from the same clade can occur in low altitudes as well (e.g. clade M2 and M3). There are, however, important mountain ranges in between the clades such as: the High-Atlas between M1 and M2 clades and, to some extent, the Anti-Atlas between M2 and M3 clades. It seems, therefore, that oreography might be a probable cause for differentiation in this species as is the case in *Buthus* scorpions (Habel et al., 2012). Vachon (1952) described two subspecies inhabiting Morocco: *A. m. mauritanicus* and *A. m. bourdoni*. The distribution we found for clade M1 largely coincides with the known distribution of *A. m. mauritanicus*, while the distribution we found for clade M2 coincides with the known distribution of *A. m. bourdoni*'s. Although Lourenço (2005) did not find evidence to support these two subspecies, the genetic distances between them are at a level of those found between full species of *Androctonus* (Table 3). In addition, clades M2 and M3 are themselves separated by a similar species-level genetic distance. An exploratory morphological analysis of the specimens of clade M3 found a very granulated prosoma and tergites, which does not fit the description of *A. mauritanicus*. These morphological characters also distinguish them from the specimens in clade M2, showing these scorpions are distinct both genetically and morphologically. A more detailed morphological assessment is clearly needed prior to a taxonomic revision.´

4.3 *Androctonus cf. liouvillei*

Near Tan Tan Plage, a coastal town, we found four unusual specimens. They are morphologically different from *A. mauritanicus and A. sergenti* Vachon, 1948 (also, dark colored species). They seem to be closer related morphologically with *A. liouvillei,* and although we only captured 4 adult male specimens, they were found to have a higher pectinal teeth count (33) than what was reported by Lourenço (2005), who reported a 28 to 32 pectinal teeth count for *A. liouvillei* males, already a broader interval than in Vachon (1952), of 28 to 30 pectinal teeth. They also have a more slender pedipalp chela of 3.6 ratio than what was reported for *A. liouvillei* by Vachon (1952), with a 3.2 ratio. No data is available in Lourenço (2005). As no other species

are known for that region, we designated the G clade specimens as *A. cf liouvillei,* a species known from this region. However, our estimate of relationships based on mtDNA clearly indicates that these are not related to *A. liouvillei* (Figure 2), although exact relationships remain poorly resolved. Further sampling will be necessary to study the relationships between this population and other species present in Morocco, but it appears to be an undescribed new species.

4.4 *Androctonus liouvillei*

The L clade is formed by *A. liouvillei* scorpions. Although this clade is sister to the *A. bicolor*, genetic distances between them are near the species-level, corroborating Lourenço's (2005) morphologically based interpretation that *A. liouvillei* is a true species and distinct from *A. bicolor*.

4.5 *Androctonus amoreuxi*

The distribution of clades A1 and A2 meet in the Erfoud area. Although there are no obvious geographical formations separating them, the genetic distance between clades is 7.5%, which is similar to the distance recovered between the *A. australis* clades. Again, as currently recognized the species appears to be paraphyletic, with clade A3 sister to *A. australis* although with very weak support levels. Although some *A. amoreuxi* specimens were separated by the largest geographic distance of any species in this study, we could not find morphological differences between them in taxonomically relevant characters (Lourenço, 2005; Vachon, 1952). The fact that the genetic distance between clades A2 and A3 is relatively high (8.9 %) can point to the validity of *A. amoreuxi*'s subspecies: *A. a. amoreuxi* in the Maghreb and *A. a. levyi* Fet, 1997 in the Sinai Peninsula. However, further morphological study of the intervening populations will be necessary prior to a taxonomic revision.

4.6 *Androctonus bicolor.*

The B clade has longer internal branches compared to other clades, but is clearly a monophyletic lineage, sister taxon to *A. liouvillei*. The most recent taxonomic survey of *Androctonus* genus estimates that *A. bicolor*, although a valid taxon, "*correspondant très probablement à une espèce polymorphe*" (Lourenço, 2005). As our samples reflect, *A. bicolor* scorpions are distributed over a large geographical distance, which might explain the deep divergences between these subclades.

Summarizing, we were able to identify cryptic variation in *A. australis*, *A. mauritanicus* and *A. amoreuxi*. Some cases were simpler, in which we could confirm previous suspicions on the validity of a distinct group, such as *A. liouvillei*. Further work should aim at sequencing nuclear genes in order to resolve deeper relationships within the genus. It would also be very interesting to obtain samples of regional species like A*. aleksandrplotkini A. hoggarensis* (Pallary, 1929), *A. maroccanus* and *A. sergenti*. Those, together with a more extensive sampling of Algeria, Libya and Mauritania could provide a prime perspective on the patterns and processes shaping *Androctonus* evolution and current distribution in North Africa.

5. Conclusions

We here show that *A. australis, A. mauritanicus* and *A. amoreuxi* have deeply divergent subclades. Such variation can be reflected in the venom these animals produce, as seen in other scorpion species (Newton et al., 2007; Omran and McVean, 2000; Smertenko et al., 2001). We therefore suggest that the venom of *A. australis, A. mauritanicus* and *A. amoreuxi* should be studied with these deep divergences in mind. Antivenom developed for scorpions in one region should be tested for efficacy against venoms from regions (Fatani et al., 2010) where scorpions correspond to different clades as identified here.

Furthermore despite lack of basal resolution, our results have clear bearing on the taxonomic status of some of the (sub)species included in our study. In addition, we identified a population of *A. liouvillei*-like specimens near Tan-Tan (clade G) which merits further study as a potential new species.

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Table 1: Geographical referencing of the sampled specimens, their codes and respective countries of origin. Coordinates are in the WGS84 datum, in decimal degrees. M.D. indicates missing data.

						Accession numbers *			
Taxon	Country	Clade	Latitude	Longitude	Sc Code	12S	16S	COI	ND1
A. amoreuxi	Morocco	A1	30,176	-6,875	304			M.D.	M.D.
A. amoreuxi	Algeria	A1	30,907	-3,997	450				
A. amoreuxi	Morocco	A1	31,143	-4,387	808				M.D.
A. amoreuxi	Morocco	A1	28,250	-9,333	1472				
A. amoreuxi	Morocco	A1	28,446	-9,373	1479				
A. amoreuxi	Morocco	A1	28,773	-9,459	1485				
A. amoreuxi	Morocco	A1	28,606	-9,430	1487				M.D.
A. amoreuxi	Morocco	A1	29,046	-8,777	1489				
A. amoreuxi	Morocco	A1	29,148	-8,605	1491				
A. amoreuxi	Morocco	A1	29,060	-8,852	1493				
A. amoreuxi	Morocco	A1	29,727	-7,975	1503				
A. amoreuxi	Morocco	A1	29,630	-8,010	1507				
A. amoreuxi	Morocco	A1	29,680	-7,982	1508				
A. amoreuxi	Algeria	A2	32,440	3,740	423				
A. amoreuxi	Algeria	A2	32,440	3,740	424				M.D.
A. amoreuxi	Morocco	A2	32,476	-1,721	791		M.D.		
A. amoreuxi	Morocco	A2	32,505	-1,502	794				M.D.
A. amoreuxi	Morocco	A2	31,143	-4,022	807				M.D.
A. amoreuxi	Tunisia	A2	33,943	8,034	913				
A. amoreuxi	Tunisia	A2	33,943	8,034	920				
A. amoreuxi	Morocco	A2	32,476	-1,721	1056				M.D.
A. amoreuxi	Morocco	A2	33,892	-2,019	1403				M.D.
A. amoreuxi	Pet trade	A3	M.D.	M.D.	627				M.D.
A. amoreuxi	Egypt	A3	M.D.	M.D.	671				M.D.
A. amoreuxi	Egypt	A3	M.D.	M.D.	770				M.D.
A. amoreuxi	Egypt	A3	M.D.	M.D.	1165				M.D.
A. amoreuxi	Egypt	A3	M.D.	M.D.	1375				M.D.
A. amoreuxi	Egypt	A3	M.D.	M.D.	1413				M.D.
A. australis	Algeria	AU1	32,440	3,740	416				M.D.
A. australis	Algeria	AU1	32,440	3,740	417				M.D.
A. australis	Algeria	AU1	32,440	3,740	418	M.D.			M.D.
A. australis	Algeria	AU1	32,440	3,740	419				M.D.
A. australis	Algeria	AU1	32,440	3,740	420				
A. australis	Algeria	AU1	32,440	3,740	421	M.D.			M.D.
A. australis	Algeria	AU1	32,440	3,740	422				M.D.
A. australis	Algeria	AU2	35,115	5,182	378		M.D.		M.D.
A. australis	Algeria	AU2	35,170	2,217	382				M.D.
A. australis	Algeria	AU2	35,368	2,055	385		M.D.		
A. australis	Algeria	AU2	35,368	2,055	386			M.D.	M.D.
A. australis	Tunisia	AU2	34,334	8,579	903				M.D.
A. australis	Tunisia	AU2	34,334	8,579	904				
A. australis	Tunisia	AU2	34,334	8,579	905	M.D.			M.D.
A. australis	Tunisia	AU2	33,943	8,034	914		M.D.		M.D.
A. australis	Tunisia	AU2	33,943	8,034	915				M.D.
A. australis	Tunisia	AU2	33,943	8,034	916				M.D.
A. australis	Tunisia	AU2	33,943	8,034	917				M.D.
A. australis	Tunisia	AU2	33,943	8,034	918				M.D.
A. australis	Tunisia	AU2	33,943	8,034	919				M.D.
A. australis	Tunisia	AU3	33,527	8,790	922				M.D.
A. australis	Tunisia	AU3	33,527	8,790	923				M.D.
A. australis	Tunisia	AU3	33,527	8,790	924				M.D.
A. australis	Tunisia	AU3	33,536	9,522	928			M.D.	M.D.

A. australis	Tunisia	AU3	33,533	9,991	932				M.D.
A. australis	Tunisia	AU3	33,533	9,991	933				M.D.
A. australis	Tunisia	AU3	33,533	9,991	934				M.D.
A. australis	Tunisia	AU3	32,785	10,373	938				M.D.
A. australis	Tunisia	AU3	32,785	10,373	939				M.D.
A. australis	Tunisia	AU3	32,785	10,373	940				M.D.
A. australis	Tunisia	AU3	33,650	10,317	948				
A. australis	Tunisia	AU3	33,650	10,317	950				M.D.
A. australis	Tunisia	AU3	33,650	10,317	951				M.D.
A. australis	Pet trade	AU4	M.D.	M.D.	626		M.D.		
A. australis	Egypt	AU4	31,279	27,055	954	M.D.			
A. australis	Egypt	AU4	31,279	27,055	957		M.D.		M.D.
A. australis	Egypt	AU4	28,809	34,228	981		M.D.		M.D.
A. australis	Egypt	AU4	28,822	34,182	992				
A. australis	Egypt	AU4	28,822	34,182	993				
A. australis	Egypt	AU4	M.D.	M.D.	1374				
A. australis	Egypt	AU4	M.D.	M.D.	1414				
A. bicolor	Algeria	B	35,208	1,541	377				M.D.
A. bicolor	Algeria	B	35,208	1,541	383			M.D.	M.D.
A. bicolor	Tunisia	B	33,846	10,128	947				
A. bicolor	Tunisia	B	33,650	10,317	949				
A. bicolor	Egypt	B	M.D.	M.D.	1093				
A. bicolor	Egypt	B	M.D.	M.D.	1371				M.D.
A. cf. liouvillei	Morocco	G	28,479	-11,212	1453				M.D.
A. cf. liouvillei	Morocco	G	28,479	-11,212	1454				
A. cf. liouvillei	Morocco	G	28,479	-11,212	1455				
A. cf. liouvillei	Morocco	G	28,479	-11,212	1456			M.D.	M.D.
A. liouvillei	Morocco	L	30,668	-6,380	210			M.D.	M.D.
A. liouvillei	Morocco	L	34,286	-3,169	780		M.D.		
A. liouvillei	Morocco	L	34,286	-3,169	781				M.D.
A. liouvillei	Morocco	L	33,892	-2,019	786				M.D.
A. liouvillei	Morocco	L	33,213	-2,019	787				M.D.
A. liouvillei	Morocco	L	32,087	-1,241	793				
A. liouvillei	Morocco	L	32,571	-2,015	796				M.D.
A. liouvillei	Morocco	L	32,571	-2,015	797				
A. liouvillei	Morocco	L	33,289	-3,780	816				M.D.
A. liouvillei	Morocco	L	33,508	-3,528	817			M.D.	M.D.
A. liouvillei	Morocco	L	33,892	-2,019	830				M.D.
A. liouvillei	Morocco	L	32,087	-1,241	839				M.D.
A. liouvillei	Morocco	L	33,213	-2,019	1055				M.D.
A. liouvillei	Morocco	L	33,892	-2,019	1207				M.D.
A. liouvillei	Morocco	L	33,892	-2,019	1394				M.D.
A. mauritanicus	Morocco	M1	32,225	-8,166	15				
A. mauritanicus	Morocco	M1	32,526	-7,863	287				
A. mauritanicus	Morocco	M1	32,661	-7,793	290				M.D.
A. mauritanicus	Morocco	M1	32,661	-7,793	291				
A. mauritanicus	Morocco	M1	32,661	-7,793	292			M.D.	M.D.
A. mauritanicus	Morocco	M1	M.D.	M.D.	625				M.D.
A. mauritanicus	Morocco	M2	30,183	-9,580	1467				
A. mauritanicus	Morocco	M2	30,183	-9,580	1468				
A. mauritanicus	Morocco	M2	30,183	-9,580	1469				M.D.
A. mauritanicus	Morocco	M2	30,159	-8,481	1549			M.D.	
A. mauritanicus	Morocco	M2	30,059	-9,084	1558				
A. mauritanicus	Morocco	M2	30,098	-8,938	1589			M.D.	M.D.
A. mauritanicus	Algeria	M3	29,068	-10,248	438				M.D.
A. mauritanicus	Morocco	M3	29,087	-9,898	1443				
A. mauritanicus	Morocco	M3	29,087	-9,898	1444				
A. mauritanicus	Morocco	M3	29,087	-9,898	1445				

Chapter III

Preface 2

Packing a pinch: functional implications of chela shapes in scorpions using finite element analysis

3.1 Preface 2

As mentioned before, scorpions appeared in the middle Silurian (425 – 450 Mya) as aquatic organisms. 100 My later, when they conquered land masses, significant morphological changes such as the shrinkage in body size and the enclosure of the external book gills followed their terrestrialization (Jeram, 2000). Since this period, between the Late Devonian and the Early Carboniferous (325 – 350 Mya), scorpions maintained the general external body plan featuring:

a) A segmented ophistosoma;
b) Mesosoma and metasoma differentiated from each other;
c) Robustly chellate pedipalps and chelicerae;
d) Eight walking legs;
e) Pectines (ventral sensory organ exclusive to Scorpions);
f) Terminal telson with a venom delivering apparatus.

Since then few changes occurred making scorpions morphologically conserved in different habitats. However specific adaptations are associated to certain environments. Lamoral (1979) and Polis (1990) identified several ecomorphotypes based on a qualitative review of the local scorpion faunas of sub-Saharan Africa and North America respectively. More recently, some of these proposed ecomorphotypes were corroborated quantitatively based on only the chela shape (see featuring article). The first comparative studies on scorpion performance traits have shown that in a sample of very few species, a clear non-phylogenetic component can be uncovered in both the morphology and performance of the chelae (van der Meijden et al., 2010, 2012). This highly suggests independent evolution of these morphotypes and thus adaptive significance for this trait. However, to study adaptive features comparatively, an estimate of the phylogeny of the included taxa is required.

The last two centuries' studies on scorpions have focused on the morphological aspects that are more informative for taxonomy. Because of that there is a great deal of morphological diversity that remains understudied. For instance, to understand the adaptive importance of morphological structures we need to look beyond taxonomy and phylogenetics. It is necessary to include functional parameters, such as performance data, and to compare them between different organisms. Comparative analyses greatly enhance our understanding of the wealth of morphological diversity that exists in the animal world and allows us to uncover historical trial-and-error based design. These studies have successfully provided insights into, for example, the ecology of the vertebrate jaw-cranial muscoskeletal system (Herrel et al., 2008). It has been one of the goals for CIBIO's Integrative Biogeography, Ecology and Evolution research group to use scorpions as a model for the evolution of associated suites of characters that are related to specific environments.

The Scorpiones is a minor order within Arachinda representing less than 2% of the number of arachnid species (Rein, 2013). However, its systematics remains, for the most part, unresolved. Competing hypothesis divide researchers (Prendini & Wheeler, 2005; Soleglad & Fet, 2003) and much debate is focused at the family level. These theories are anchored in cladistic approaches based mostly in morphological traits but they are open for future findings with DNA sequence data. With the introduction of DNA sequence data, several authors attempted to solve high-level scorpion relationships (*e.g.* Hassanin, 2006; Masta *et al.*, 2008). The use of molecular markers has proven more efficient at lower taxonomical levels. The fast emergence of molecular phylogenetic data on scorpion genera (*e.g.* Fet *et al*., 2003a; Prendini *et al*., 2003) and species (*e.g. Euscorpius*: Fet *et al*., 2003b; *Buthus* Sousa *et al*, 2011; *Hottentotta*: Sousa *et al*., 2010; *Mesobuthus*: Gantanbein *et al*., 2003) is a new challenge for scorpion taxonomy.

Packing a pinch: functional implications of chela shapes in scorpions using finite element analysis represents some of the work that the candidate developed as a collaborator. Under the supervision of Dr. Arie van der Meijden, also the lead author, the candidate assisted with the measuring of bite forces. Bite force measurement consists of the record of several trials per specimen of scorpion's chelae pinch forces. The recordings are made using a custom built force sensor (further details are given in

the *Materials and Methods* section). The candidate was able to work under a climate-controlled room either in the laboratory or in field conditions. Field measurement is an essential part of this work because we could make records with animals that were removed from natural conditions in the same day. Although scorpions perform well under captivity, field measurements are also useful to record scorpions that cannot be obtained from the pet trade.

As mentioned above, to retrieve the phylogenetic signal from the evolutionary history of the studied traits we sequenced 20 scorpions of 8 families. 12S, 16S and COI mitochondrial genes were used to build the phylogenetic tree. All the molecular laboratory protocols were performed by the MSc candidate following the same methodology applied for sequencing *Androctonus* scorpions. The MSc's contribution did not included morphological measurements, specimen scanning or any data analysis. The candidate wrote part of the *Phylogenetic analysis* section of *Materials and methods,* while the remainder of the article was written by Arie van der Meijden and Dr. Thomas Kleinteich.

The produced work was accepted for publication in the *Journal of Anatomy* at the 26th January, 2012.

3.2 Packing a pinch; Functional implications of chela shapes in scorpions using finite element analysis.

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Running title: Comparative FEA of scorpion chelae

Abstract

Scorpions depend on their pedipalps for prey capture, defense, mating and sensing their environment. Some species additionally use their pedipalps for burrowing or climbing. Because pincers or chelae at the end of the pedipalps vary widely in shape, they have been used as part of a suite of characters to delimit ecomorphotypes. We here evaluate the influence of the different chela cuticular shapes on their performance under natural loading conditions. Chelae of 20 species, representing seven families and spanning most of the range of chela morphologies, were assigned to clusters based on chela shape parameters using hierarchical cluster analysis. Several clusters were identified to approximately correspond to described scorpion ecomorphotypes. Finite element models of the chela cuticulae were constructed from CT scans and loaded with estimated pinch forces based on in vivo force measurements. Chela shape clusters differed significantly in mean Von Mises stress and strain energy. Normalized FEA showed chela shape to significantly influence Von Mises stress and strain energy in the chela cuticula, with Von Mises stress to vary up to an order of magnitude, and strain energy varying up to two orders of magnitude. More elongate, high-aspect ratio chela forms showed significantly higher mean stress than more robust low-aspect ratio forms. This suggests that elongate chelae are at a higher risk of failure when operating near the maximum pinch force. Phylogenetic independent contrasts (PIC) were calculated based on a partly resolved phylogram with branch lengths based on an alignment of the 12S, 16S and CO1 mitochondrial genes. PIC showed cuticular stress

and strain in the chela to correlate with several shape parameters, such as aspect ratio, movable finger length, and chela height, independently from phylogenetic history. Our results indicate that slender chela morphologies may be less suitable for high-force functions such as burrowing and defense. Further implications of these findings for the ecology and evolution of the different chela morphologies are discussed.

Key words

Finite element analysis, Scorpiones, independent contrasts

Introduction

Scorpions are a large and ancient group of chelicerates with close to 2000 described species (Fet et al., 2000), which have successfully adapted to a diversity of habitats on all continents except Antarctica. Despite occurring in a wide variety of habitats, scorpions have changed little in overall body plan since the Silurian (Dunlop et al., 2008). Their most notable features are the pedipalps carrying the chelae (the “pincers”), and the metasoma (the “tail”) carrying the venomous telson (the “stinger”). All these structures are used in prey subjugation and defense. The pedipalps are additionally used in mating, climbing, digging, and serve as a sensory array similar to the antennae of insects and crustaceans (Alexander, 1959; Fet et al., 2003).

The chelae are formed by the last two segments of the pedipalps; the manus or tibia which contains the muscles and which also forms the immovable finger, and the movable finger or tarsus. The movable finger is adducted by three muscle bundles in the chela manus (Gilai & Parnas, 1970). In addition, there is also a small closing muscle in the next segment (patella), which is connected to the movable finger by a long ligament (Snodgrass, 1952; Gilai & Parnas, 1970). The movable finger is abducted by the elastic recoil of resilin in the joint (Govindarajan & Rajulu, 1974; Sensenig & Shultz, 2004), opening the chela. The movable finger rotates around an axis defined by two joints, located on the median and lateral sides of the chela.

Despite conservancy in their ecological role as mostly terrestrial predators, scorpions have been known to have specific adaptations to certain environments. Several ecomorphotypes were recognized based on a qualitative review of the local scorpion faunas of sub-Saharan Africa (Lamoral, 1979) and North America (Polis, 1990). In these studies, the shape of the chelae is considered an important character in the delimitation of five putative ecomorphotypes. Robust chelae with a high muscle-filled manus and relatively shorter fingers that are reminiscent of chelae in durophagous crabs are ascribed to fossorial (digging) species. Sand-dwelling (psammophilous)

species have more elongate chelae with long fingers. Species that hide in or under rocks (saxicoline) or bark (corticoline) have dorso-ventrally compressed bodies and appendages including the chelae, to facilitate living in narrow spaces (Newlands, 1972). Actively foraging scorpions with slender bodies and appendages are sometimes considered as an additional ecomorphotype; the errant ecomorphotype (Polis, 1990). Chelae of the errant ecomorphotype are similar to the chelae in psammophilous species. These different putative ecomorphotypes have never been quantitatively corroborated.

The described different morphologies change the functional properties of the chelae. The volume of the manus is largely determined by the amount of muscle in that segment, which partly determines the force a scorpion can produce with its chelae. Like in durophagous crabs (Yamada & Boulding, 1998), the height of the manus relative to the length of the chela is therefore indicative of pinch force (Van der Meijden et al., 2010). The pinch force of a scorpion is further determined by the lever system that transfers the force from the muscles to the tips of the fingers of the chelae. The mechanical advantage of this lever system is very variable in scorpions. As the outlever (the external part of the movable finger) of a first class lever system gets longer relative to the inlever (the internal part of the movable finger, to which the muscles attach), the mechanical advantage of the lever system may increase speed at the cost of force (but see McHenry, 2011). Longer fingers may thus give the scorpion an advantage to catch elusive insect prey by increased closing speed or gape size. Since this arrangement makes the chelae relatively inefficient in producing high forces, such long-fingered chelae are feeble weapons to fend of predators. The robust chelae of fossorial species allow for more muscle to be packed into the chela and for a longer inlever inside the chela manus, thus improving the mechanical advantage for force production. Newlands (1969; 1972) suggested that the large chelae may also be used in blocking the burrow, to keep out predators. Other authors have noted that scorpions with robust chelae use their sting less in prey incapacitation than scorpions with more slender chelae (Stahnke, 1966; Baerg, 1961; McCormick & Polis, 1990), which suggests a trade-off between the chelae and the stinger in prey incapacitation. Although morphological characters of the chela are important in taxonomic studies, the functional implications of shape to the performance of the chela have received little attention. Knowledge on the functional consequences of different chela shapes, however, is crucial to understand their adaptive value and to identify functional demands during scorpion evolution. We here for the first time evaluate the functional implications of the shape of the chela cuticula in scorpions. We use 3-dimensional Finite Element Models to

estimate the stresses that the chela cuticula of different chela morphologies experience under biologically realistic force production.

Materials and Methods

Species selection

A total of 20 species (Table 1) was selected from seven families to represent the range of chela morphology found in extant scorpions. Animals were selected based on availability through field collecting or pet-trade.

Morphological measurements

Morphological measurements were taken using digital calipers. Several preserved specimens were measured per species and average chela measurements were calculated. For some species however, only a single specimen was available. Morphological data are shown in table 1. In addition to linear measurements, also the curvature of the movable finger was determined using a custom Matlab script (available from the corresponding author upon request). The ventral view of the chela movable finger was used to fit a circle to the inside and outside curve of the movable finger. The average radius of these two circles was then divided by the length of the movable finger to attain a size-independent metric of curvature. In addition the angle between the axis of rotation of the movable finger to the line connecting the tip of the movable finger to the center of the axis of rotation was calculated (α in figure 1c). The angle between the in-lever and out-lever of the movable finger was calculated as the angle between the plane defined by the finger tip (T), the median joint (MJ) and lateral joint (LJ), and the plane defined by the insertion point of the muscles (MI), the median joint (MJ) and lateral joint (LJ) (see figure 1a).

In order to group similarly shaped chelae, we performed a clustering analysis on normalized linear measurements of the chela. Manus height, manus width and length of movable finger were normalized by division with total chela length. These normalized data were used to identify clusters of similarly shaped chelae. Clustering analysis was performed in R (R core development team). The data were clustered hierarchically using the Ward method based on Euclidian distance between species. Clusters in the data were also identified using the k-clustering method, with the variable k (number of clusters), identified through the expectation maximization algorithm as implemented in the R package “Mclust”.

Table 1. Species used in this study, and measured and derived morphological parameters. Asterisk indicates measurements from a similar sized specimen.

Species		Family	Measured specimens	Total length (mm)	Prosoma length (mm)	Chela length (mm)	Normalized width	Normalized height	Normalized movable finger length	Aspect ratio	Mechanical advantage	Angle in-lever-out-lever (degrees)	Angle out-lever – axis of rotation (degrees)	Measured max force (N)	Predicted force (N)	Curvature movable finger	Max. Von Misses stress (Pa) – scaled	Mean Von Misses stress (Pa) – scaled	Total strain energy (J) corrected	12S accession number	Accession number 16S	Accession number CO1
Androctonus amoreuxi	(Audouin, 1826)	Buthidae	8	81.8	10.21	19.45	0.24	0.27	0.64	3.34	4.69	101.2	77.0	2.0*	3.75	2.35	1.43E+08	3.99E+06	1.67E-06	JQ423120	JQ514228	JQ514246
Androctonus australis	(Linnaeus, 1758)	Buthidae	15	78.4	10.12	19.02	0.28	0.31	0.66	3.53	4.29	85.8	80.6	2.8*	3.3	1.81	1.15E+08	5.04E+06	1.78E-06	JQ423124	JQ514232	JQ514250
Bothriurus chilensis	(Molina, 1782)	Bothriuridae	1	35.0	4.39	6.18	0.31	0.38	0.53	2.53	3.56	56.4	79.5		0.53	1.86	1.06E+08	2.10E+06	4.14E-07	Missing	JQ514230	Missing
Caraboctonus keyserlingi	Pocock, 1893	Iuridae	8	35.6	5.84	8.66	0.32	0.34	0.58	3.02	4.35	104.3	63.2		0.91	2.50	2.30E+08	3.53E+06	1.18E-06	JQ423123	JQ514231	JQ514249
Chactas sp.		Chactidae	1	36.5	6.51	10.58	0.36	0.50	0.60	2.07	3.18	66.7	70.2		2.1	1.75	3.20E+07	1.85E+06	2.78E-07	JQ423128	JQ514239	JQ514255
Euscorpius flavicaudis	(DeGeer, 1778)	Euscorpiidae	1	26.7	4.92	8.44	0.22	0.37	0.57	3.56	3.89	100.7	56.4		0.41	1.91	3.92E+07	2.04E+06	4.92E-07	JQ423126	JQ514237	JQ514253
Grosphus flavopiceus	Kraepelin, 1900	Buthidae	10	105.2	11.18	20.55	0.26	0.26	0.59	3.85	5.01	124.3	79.7	0.9	3.66	1.61	9.54E+07	3.92E+06	2.02E-06	JQ423127	JQ514238	JQ514254
Hadogenes paucidens	Pocock, 1896	Liochelidae	12	130.5	14.63	26.83	0.18	0.34	0.50	2.96	3.24	107.9	57.4	18.2	12.92	2.17	7.32E+08	3.14E+06	7.53E-07	JQ423130	Missing	JQ514257
Hadrurus arizonensis	Ewing, 1928	Iuridae	9	86.9	12.69	21.1	0.22	0.34	0.70	3.13	5.4	76.4	78.8	3.4*	7.83	2.20	1.43E+08	4.65E+06	2.33E-06	JQ423129	JQ514240	JQ514256
Hoffmannius sp.		Vaejovidae	1	33.2	4.36	6.99	0.17	0.19	0.70	5.64	7.5	62.4	83.1		0.09	2.45	1.14E+07	7.71E+06	9.14E-06	JQ423133	JQ514243	Missing
Hottentota gentili	(Pallary, 1924)	Buthidae	10	85.8	9.94	20.59	0.18	0.19	0.69	4.49	6.03	106.4	82.3	1.2*	1.84	2.05	4.34E+08	7.88E+06	5.95E-06	JQ423119	JQ514227	JQ514245
Iurus dufoureius	(Brullé, 1832)	Iuridae	1	64.8	10.01	20.95	0.26	0.34	0.61	3.03	4.03	71.9	72.5		4.21	1.93	2.85E+08	2.95E+06	6.69E-07	JQ423125*	JQ514235*	JQ514252*
Leiurus quinquestriatus	Ehrenberg, 1828	Buthidae	9	75.8	9.22	17.22	0.15	0.17	0.72	6.55	7.52	64.6	82.3	0.7*	0.48	2.03	2.28E+08	1.51E+07	1.44E-05	JQ423131	JQ514241	JQ514258
Liocheles australasiae	(Fabricius, 1775)	Liochelidae	1	21.6	4.02	7.3	0.18	0.37	0.42	2.81	3	55.5	66.7		0.35	1.42	2.04E+07	2.30E+06	3.79E-07	Missing	JQ514233	DQ127506
Opisthacanthus madagascariensis	Kraepelin, 1894	Liochelidae	7	42.8	7.46	14.31	0.23	0.42	0.54	2.31	3.02	97.8	59.4	2.9*	2.71	1.84	4.13E+08	1.41E+06	1.89E-07	Missing	JQ514236	Missing
Opistophthalmus boehmi	(Kraepelin, 1896)	Scorpionidae	6	48.2	7.38	11.69	0.29	0.50	0.59	2.16	3.28	72.6	70.2		2.86	1.89	5.41E+07	1.79E+06	2.13E-07	JQ423122	Missing	JQ514248
Orthochirus innesi	Simon, 1910	Buthidae	1	30.0	3.8	5.04	0.18	0.20	0.65	5.18	5.09	62.1	86.0		0.08	2.73	7.04E+07	4.57E+06	3.00E-06	JQ423118	JQ514226	JQ514244
Pandinus cavimanus	(Pocock, 1888)	Scorpionidae	3	81.6	15.74	26.5	0.28	0.62	0.66	1.45	3.1	54.1	75.8	24.5	36.5	2.15	1.96E+08	1.92E+06	2.06E-07	AY156550	JQ514234*	JQ514251*
Parabuthus transvaalicus	Purcell, 1899	Buthidae	5	88.0	9.38	13.56	0.23	0.24	0.66	4.99	5.82	74.8	83.2	0.4	1.18	2.51	7.78E+07	4.25E+06	2.01E-06	JQ423121	JQ514229	JQ514247
Scorpio fuliginosus	(Pallary, 1928)	Scorpionidae	8	n.a.	8.34	11.88	0.33	0.64	0.57	2.89	2.54	62.8	67.9	6.3*	2.7	1.57	1.93E+08	1.09E+06	1.69E-07	JQ423132	JQ514242	JQ514259

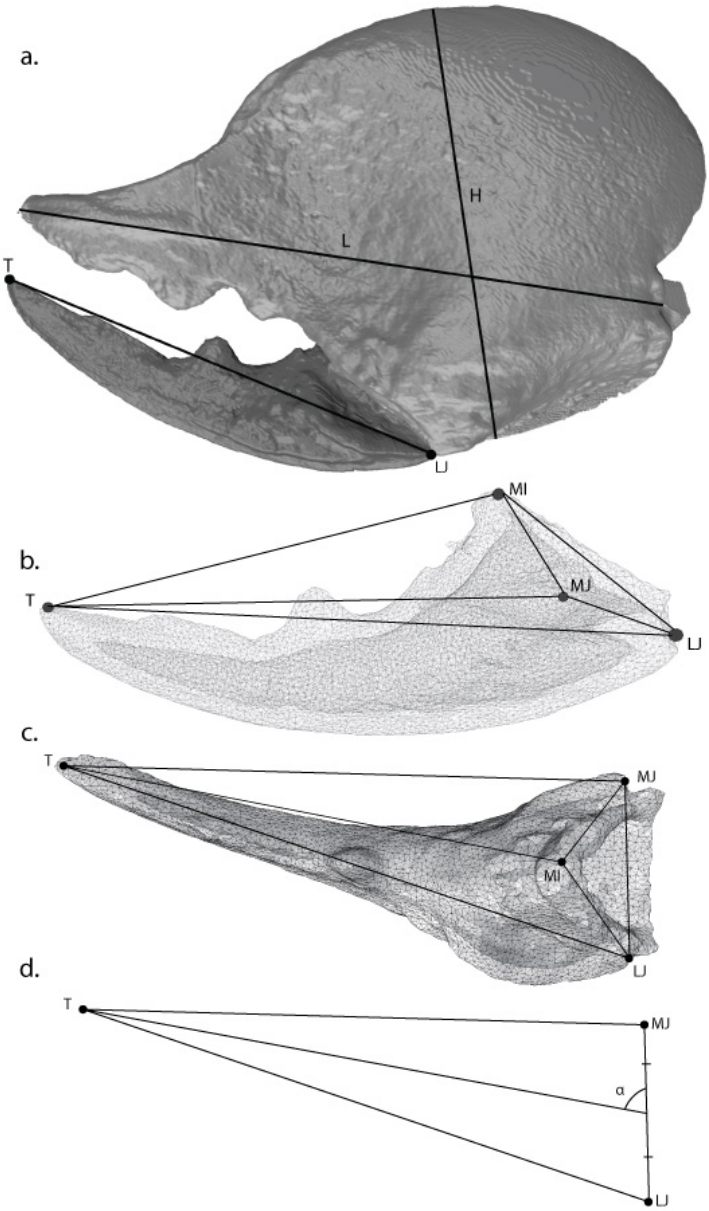

Figure 1. a; Linear measurements on the chela Length (L), Height (H), movable finger length (T-LJ). b-c; Measurements on chela movable finger, exemplified on the movable finger of *Pandinus cavimanus*. a. Side view of a transparent wireframe mesh. b. Dorsal view, solid wireframe mesh. c. Dorsal view showing angle (α) between center of joint axis (LJ-MJ) and finger tip (T). LJ; lateral joint, MJ; medial joint, MI: muscle insertion, T; tip. The line LJ-MJ is the axis of rotation for the movable finger.

Pinchforce measurement and estimation

Live animals were maintained as described in Van der Meijden et al. (2010). *In vivo* pinch forces were measured using either a Kistler force transducer (type 9203, Kistler Inc., Switzerland) mounted on a purpose-built holder (see Herrel et al., 1999), or using a similar setup with a Sauter FH20 external force sensor (Sauter ltd., Germany). Measurements were made in a climate-controlled room at 23-24˚C. During pinch-force measurements, scorpions were restrained between sponge pads in which a cutout was made to accommodate the body, or by placing a padded clamp over the last segments

of the metasoma to allow safe handling. Most specimens pinched readily, but some were stimulated to pinch by stroking the inside of the chela with the tip of a pair of tweezers. Five trials were performed, separated by at least one day. Only the maximum force per chela was retained for further analyses.

Not for all species included in this study were live specimens available to measure pinch forces. Further, some species were too small for accurate measurements using parallel plates. For species for which *in vivo* pinch forces were not available, we estimated pinch forces based on the aspect ratios of the chelae. Chela aspect ratio was previously demonstrated to be a good predictor for pinch forces (R^2 = 0.90; Van der Meijden et al. 2010). We further expanded the dataset from Van der Meijden et al. (2010) to comprise 170 specimens from 20 species, representing four families (Buthidae, Iuridae, Liochelidae, Scorpionidae; data to be published elsewhere). The maximum pinch forces were log10 transformed, and standardized for the size of the animal by regression of the forces against log10 transformed prosoma length. Body mass or total length cannot be used as a proxy for overall size in scorpions, as both body mass and total length can change significantly depending on feeding state. The residuals were then regressed against the aspect ratio of the chela (length/height). Pinch force was predicted based on a line function that was fit to the data using least squares (R^2 = 0.54, Pearson's correlation coefficient -0.73). The resulting predicted maximum pinch forces are shown in table 1.

Specimen scanning and model reconstruction

We CT scanned the chelae of the specimens examined herein with a Skyscan 1076 micro CT scanner that was available at the Small Animal Tomographic Analysis Facility at the Seattle Children's Research Institute in Seattle, WA, USA. The specimens were scanned at a source voltage of 31 kV and a source currency of 187 µA at 35 µm resolution. The raw x-ray images that were recorded during CT imaging where then converted with the software tool *NRecon* (Skyscan) into a volumetric data set that consisted of a stack of TIFF image files.

The volumetric dataset was imported into the 3D visualization software Amira 5.3.3 (Visage Imaging). In Amira, we used the *Labels* function to separate the cuticles of the scorpion chelae from the remainder structures that where visible in the CT data set. We further separated the cuticle of the manus from the cuticle of the movable finger and saved them as two separate materials in the *Labels* dataset that is generated during segmentation in Amira. Based on the materials in the *Labels* dataset, we calculated polygonal surfaces for the manus and the movable finger with Amira. The resulting

surfaces were then edited in the *Surface Editor Mode* of Amira to equally distribute the polygons over the surface, to reduce the total number of polygons, and to fix occurring intersections between polygons that were in close proximity to each other. For each specimen, we measured the surface area and the volume with Amira. We then converted the polygonal surfaces to solid models that were build from tetrahedral elements, i.e. the elements were defined by four nodes. Numbers of elements ranged from 20,000 to 2.4 million depending on the size of the specimen (Table 2).

Finite Element Analysis

The solid models for the manus and the finger of each specimen were imported as IDEAS files into the Finite Element modeling and analyzing software Marc Mentat 2005 R3 (MSC Software). Because the gapes between manus and finger in different specimens differed, we had to manually adjust the gape angles in Marc Mentat. For this, we oriented the models to align the axis that connects the centers of the two joints between manus and finger with the origin of the coordinate system in Marc Mentat. This allowed us to rotate the manus and the finger independently without dislocation at the joint and to set the gape angle at 15° for each specimen. For a more realistic estimate on the reaction forces that act on the joint between manus and finger under load we declared elements of the manus and of the finger to be on two different *Contact Bodies* and we assigned the nodes in the joint area as *Contact Areas*. Within *Contact Areas*, Marc Mentat treats elements that are closer to each other than a defined threshold value (1/20 of the size of the smallest element) as contacting elements. For the finite element analysis, the contact between finger and manus was treated as two-sided (i.e. the finger contacts the manus and vice versa) and we used the *Optimized Contact Constraints* option to avoid penetration between finger and manus.

We treated the cuticle of the chelae as isotropic material with a Young's modulus of 7 GPa and a Poisson's ratio of 0.3. Young's moduli of arthropod cuticle have been reported to cover a wide range from 0.1 GPa to 20 GPa (Vincent & Wegst, 2004). Although the value for the Young's modulus of 7 GPa that we have chosen herein is only a estimate based on the assumption that scorpion chelae have a rather hard cuticle and absolute output values are biased by this estimate, finite element modeling provides a powerful tool for comparisons between species, even if knowledge on the exact material attributes is not available (Dumont et al., 2009).

Table 2. Details of the chela models used in this study.

Species	Modeled Chela	# Elements Manus	# Elements finger	Surface area Manus (m^2)	Surface area finger (m^2)	Total surface area (m^2)	Force : total surface area (N m^{-2})	Surface scaling factor	Scaled force (N)	Volume manus (m^3)	Volume finger (m^3)	Total volume (m^3)	Scaled force : total volume$^{1/6}$ (N $m^{-3/6}$)	Energy scaling factor
Androctonus amoreuxi	Left	602.606	329.656	3.54E−04	7.42E−05	4.28E−04	9.21E+03	0.284	1.07	4.54E−08	1.97E−08	6.51E−08	16.83	0.103
Androctonus australis	Left	473.202	339.943	3.36E−04	8.59E−05	4.22E−04	9.49E+03	0.329	1.08	4.07E−08	1.66E−08	5.73E−08	17.44	0.100
Bothriurus chilensis	Left	178.886	58.392	5.33E−05	9.45E−06	6.27E−05	9.52E+03	0.305	0.16	2.79E−09	6.71E−10	3.46E−09	4.14	0.419
Caraboctonus keyserlingi	Left	76.686	28.877	8.50E−05	1.62E−05	1.01E−04	9.00E+03	0.285	0.26	5.12E−09	1.12E−09	6.24E−09	6.07	0.286
Chactas sp.	Left	225.370	109.687	1.74E−04	2.90E−05	2.03E−04	1.30E+04	0.246	0.52	1.51E−08	3.87E−09	1.90E−08	10.03	0.173
Euscorpius flavicaudus	Right	127.830	43.706	8.10E−05	1.49E−05	9.59E−05	8.76E+03	0.605	0.25	6.02E−09	1.31E−09	7.33E−09	5.69	0.305
Grosphus flavopiceus	Right	732.718	301.907	3.40E−04	6.55E−05	4.05E−04	9.71E+03	0.284	1.04	5.38E−08	1.49E−08	6.87E−08	16.27	0.107
Hadogenes paucidens	Right	881.421	375.594	7.78E−04	1.35E−04	9.13E−04	1.20E+04	0.182	2.35	1.37E−07	3.40E−08	1.71E−07	31.53	0.055
Hadrurus arizonensis	Left	786.112	460.487	3.66E−04	1.07E−04	4.73E−04	6.54E+03	0.155	1.22	4.71E−08	1.92E−08	6.63E−08	15.72	0.110
Hoffmannius sp.	Left	18.582	6.795	2.35E−05	3.94E−06	2.74E−05	4.32E+03	0.787	0.07	1.88E−09	2.34E−10	2.11E−09	2.04	0.850
Hottentota gentili	Right	831.107	305.133	2.74E−04	7.93E−05	3.53E−04	6.28E+03	0.487	0.90	3.33E−08	1.26E−08	4.59E−08	15.00	0.116
Iurus dufoureius	Left	906.933	476.716	4.56E−04	1.07E−04	5.63E−04	1.05E+04	0.344	1.45	7.07E−08	1.98E−08	9.05E−08	21.61	0.080
Leiurus quinquestriatus	Left	129.136	97.081	1.40E−04	4.44E−05	1.84E−04	3.71E+03	1.000	0.48	1.05E−08	4.53E−09	1.50E−08	9.60	0.181
Liocheles australasiae	Right	59.974	18.467	6.30E−05	9.49E−06	7.24E−05	1.00E+04	0.545	0.19	2.95E−09	6.77E−10	3.63E−09	4.91	0.354
Opisthacanthus madagascariensis	Right	410.352	254.362	2.76E−04	4.42E−05	3.20E−04	1.20E+04	0.303	0.82	3.23E−08	1.02E−08	4.25E−08	13.92	0.125
Opistophthalmus boehmi	Left	210.280	185.500	1.88E−04	3.96E−05	2.27E−04	1.14E+04	0.208	0.60	2.32E−08	6.05E−09	2.93E−08	10.72	0.162
Orthochirus innesi	Left	14.562	5.616	1.47E−05	4.15E−06	1.89E−05	5.16E+03	0.659	0.05	6.00E−10	2.43E−10	8.43E−10	1.74	1.000
Pandinus cavimanus	Left	1.671.634	637.596	1.47E−03	3.64E−04	1.83E−03	1.04E+04	0.129	4.71	2.71E−07	8.02E−08	3.51E−07	56.02	0.031
Parabuthus transvaalicus	Right	374.713	160.650	1.75E−04	2.29E−05	1.98E−04	3.93E+03	0.329	0.39	1.12E−08	4.48E−09	1.57E−08	7.78	0.223
Scorpio fuliginosus	Left	919.610	211.896	5.40E−04	6.68E−05	6.07E−04	7.89E+03	0.376	1.02	4.69E−08	9.29E−09	5.62E−08	16.41	0.106

We applied the following constraints (in Marc Mentat *Boundary Conditions*) to the models: (1) all nodes in the areas of muscle attachment on the manus and the finger where fixed to prevent translation or rotation for each degree of freedom; (2) bite force was distributed over four nodes on each, the finger and the manus, and modeled as point loads. We ran two separate analyses for each specimen. (1) We used the measured and predicted force values in Table 1 as bite force (absolute approach with unscaled results) and (2) we calculated the force per surface area ratios for each specimen and applied bite forces that were scaled to fit the smallest force per surface area ratio in our specimen sample (comparative approach with scaled results).

We used three different measures to quantify the biting performance of scorpion chelae with Finite Element Analysis: (1) maximum Von Mises stress, (2) mean Von Mises stress, and (3) total strain energy. Von Mises stress is used as a measure for how close a material is to failure. Areas in the chelae with higher Von Mises stresses are closer to failure than areas that experience lower Von Mises stresses. However, maximum Von Mises stresses can be hard to interpret in Finite Element models because the constrained areas (i.e. the applied point loads and fixed nodes) tend to show artificially high Von Mises stresses (Dumont et al., 2009). Mean Von Mises stress is supposed to be a more reliable measure of overall performance based on the assumption that chelae shapes that perform better under load will show lower Von Mises stresses over each node than shapes that perform poorly. Total strain energy reflects the amount of work that is used for deformation of the shapes, rather than for biting. It is assumed that scorpions that perform better during biting exhibit lower total strain energies, i.e. their chelae deform less under load. Von Mises stress and total strain energy are standard outputs of Marc Mentat.

Because in the comparative approach, all models had identical force to surface area ratios, Von Mises stresses were directly comparable between species (Dumont et al., 2009). However, total strain energy scales with the volume of the models instead of the surface area. To make the calculated values for total strain energy directly comparable between species, we calculated an energy scaling factor that was based on the specimen with the lowest force per sixth root of the volume ratio. Volume corrected total strain energies where then calculated by multiplication of the square of the energy scaling factor with the raw total strain energies. A detailed derivation of the equations to scale finite element models was provided by Dumont et al. (2009).

The numbers of elements for each specimen varied notably despite similar edge lengths of the elements (Table 2). This was caused by the different sizes of the specimens. To estimate the effect of mesh-size on the performance parameters

evaluated herein, we re-meshed the finite element model of the specimen with the least amount of elements (*Orthochirus innesi*), to generate models with eight, respectively 64 times the number of the original elements (Supplementary material). Finite element analysis with the re-meshed datasets showed only slight variations in mean Von Mises stress and total strain energy that are negligible compared to the interspecific variation. This indicates that mean Von Mises stress and total strain energy can be used for comparisons among datasets that consist of different amounts of elements; max. Von Mises stress increases in the re-meshed models, which is likely to be caused by an increased localization of point loads.

Phylogenetic analysis and independent contrasts

Total DNA was extracted from fresh or preserved (96% ethanol) muscle tissue using standard high-salt protocols (Bruford et al., 1992). A fragment of the mitochondrial cytochrome C oxidase, subunit I (*COI*) gene was amplified by PCR using primers LCO1490 and HCO2198 (Folmer et al., 1994) or primers in the same locus based on an alignment of *CO1* sequences available in GenBank as of 4-2011. The sequence of these primers was: forward (5'-WTYCTACIAATCAYAARGATATTGG-3') and reverse (5'-TAMACYTCIGGGTGWCCAAAAAAYCA-3'). A fragment of the mitochondrial *16S* rDNA gene was amplified using the primers LR-J-12887 (Simon et al., 1994) as forward primer, and a scorpion-specific reverse primer (Gantenbein et al., 2000). *12S* primers were designed based on available *12S* sequences in GenBank: forward primer 12S_F_AvdM (5'-AGAG-TGACGGGCAATATGTG-3)' and reverse primer 12s_r_AvdM (5'-CAGCGGCTGCGGTTATAC-3').

Purified PCR templates were sequenced using dye-labeled dideoxy terminator cycle sequencing on an ABI 3130 automated DNA sequencer or on an ABI 3730XL at Macrogen Inc. using the corresponding PCR primers. Chromatograms were checked and when necessary corrected using FinchTV, version 1.4.0 (Geospiza, Inc.; USA; http://www.geospiza.com). The obtained DNA sequences were aligned using MEGA 5 (Tamura et al., 2011). The coding sequence of *CO1* was aligned based on the translated amino acid sequence, and *12S* and *16S* rRNA sequences were aligned using Muscle (Edgar, 2004) as incorporated into MEGA 5 using the default settings.

Two methods of phylogenetic analysis, maximum likelihood (ML) and Bayesian Inference (BI), were conducted using PhyML, version 3.0.1 (Guindon & Gascuel, 2003) and MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) respectively. The best fit models of nucleotide evolution were determined under the Akaike information criterion in JModeltest 0.1.1 (Posada, 2008). Nodal support for the topologies recovered in the ML

analyses was obtained with 1000 bootstrap replicates. The BI analyses were run with 5,000,000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 125,000 trees). Log likelihood scores for the remaining trees were graphed in Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer) and checked for appropriateness of the burnin-period.

Phylogenetic analysis of our molecular dataset did not contradict the current taxonomic relationships. Since phylogenetic reconstruction based on the combined alignment did not resolve all interfamilial relationships, for further analysis relationships between family level clades were changed to a star topology (by setting those branch lengths to zero). We grouped members of the same family together based on the taxonomy provided by Prendini and Wheeler (2005). Since our phylogenetic reconstruction of the relationships within the family Buthidae were well resolved and received high bootstrap support, we did not make any changes within the Buthidae. The full alignment was then used to produce a ML estimate (GTR+I+G) of the branch lengths with MEGA 5. The resulting phylogram was used to calculate phylogenetic independent contrasts of chela shape measurements and performance parameters using Phylocom (Webb et al., 2008). To control for an effect of the lack of support for interfamilial relationships, we also performed an independent contrast analysis on only the representatives of the Buthidae family, for which our molecular data provided good support.

Results

Clustering analysis

The expectation maximization algorithm found 8 clusters with 2 or 3 species per cluster in the normalized linear chela measurements, showing significant structure in the dataset. All data clustering results are shown in figure 2. The chela shape data divide the dataset in two equal sized clusters, named cluster a and b. These clusters are further subdivided, and five of the subclusters were named a1-a3 and b1-b2. These clusters were not those selected using the expectation maximization algorithm (which selected 8 smaller clusters), but more inclusive clusters were chosen as the authors believe them to better correspond with described ecomorphological types of chela morphology. Mann-Whitney tests were conducted to test differences in morphological and performance related traits between all named clusters. The results of these tests can be found in table 3. Of the normalized chela measurements that were used to define the clusters, only chela width did not significantly differ between the major clusters a and b. Subcluster b2, corresponding roughly with Lamoral's (1979)

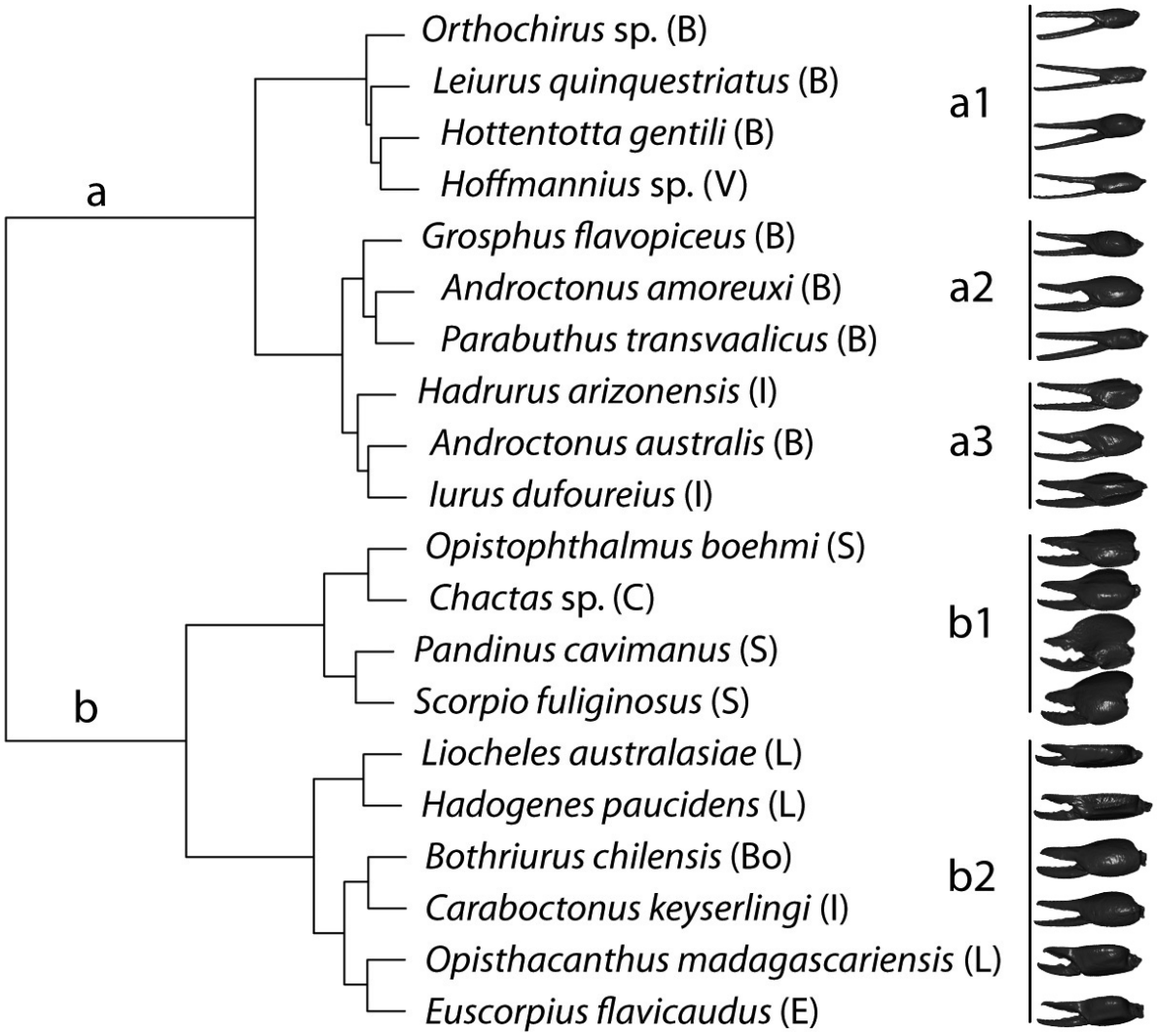

Figure 2. Hierarchical clustering of chela shapes based on movable finger length, manus width and height, normalized for chela length. Cluster a contains the more elongate chela morphologies, cluster b contains the more robust chelae. Clusters a1, a2 and a3 get progressively lower aspect ratios. Cluster b1 contains short strong chelae, whereas cluster b2 contains more flattened morphologies, typical of species that live in rock crevices and under bark.

infrasaxicolous, lithosaxicolous and infracorticolous types, differed significantly from all other subclusters in chela height and movable finger length. There was no statistical difference between any of the clusters in chela length relative to the length of the prosoma (the latter being a good indicator of overall size).

Finite element analysis

Absolute approach. Under realistic loading conditions (i.e. the applied point loads corresponded to either actually measured *in vivo* bite forces or bite force estimates based on chela aspect ratio) maximum Von Mises stress, mean Von Mises stress, and total strain energy differed between species by several orders of magnitude (Table 1).

This is in part accounted for by the different sizes of the specimens. Maximum Von Mises stress was highest in *Hadogenes paucidens* (7.32e8 Pa) and lowest in *Liocheles australasiae* (3.76e7 Pa). Mean Von Mises stress varied between 2.89e6 Pa in *Scorpio* and 3.64e7 Pa in *Hadrurus arizonensis*. Total strain energy was lowest in the very small *Orthochirus* (7.62e-6 J) and highest in the largest specimen included in this study, *Pandinus cavimanus* (1.25e-2 J).

Comparative approach. Scaling of the models based on surface area (for Von Mises stress), respectively volume (for total strain energy), allowed us to remove the effects of the size of the specimens from the analysis and to reveal the impacts of the different shapes on the performance. In our sample, the short fingered species *Scorpio fuliginosus* and *Ophistacanthus madagascariensis* showed the least overall deformation under load, expressed in the lowest values for total strain energy. Contrary, species with elongated fingers like *Hoffmannius* sp. and *Leiurus quinquestriatus* showed the most pronounced deformations of the chelae. The same pattern also emerges for mean Von Mises stress; *L. quinquestriatus* encounters highest mean Von Mises stresses and *Scorpio* shows the lowest values. Maximum Von Mises stress is highest in *Hadogenes paucidens* and lowest in *Hoffmannius*. This however is most likely caused by the presence of artificially high point loads in some of our models and the pattern of highest stresses in *H. paucidens*, and lowest stresses in *Hoffmannius* is not reflected by the plots of Von Mises stress over the 3D surfaces (Figure 3).

The fingers of the chela act under load like a beam, i.e. they experience tensile stress along the edge where the load is applied and stress by compression along the edge opposite the load; between the compressed and tensioned edges lies a neutral axis with low stresses (Figure 3). In species with slender, elongated fingers, the stresses on the movable part are similar to the counteracting fixed finger. In species with short chelae (e.g. *Scorpio*, *Pandinus cavimanus*), the roughly triangular shape of the fixed finger in lateral view tends to reduce the stress by compression on the edge opposite to the load. This results in an asymmetric stress distribution with higher stresses in the movable finger, compared to the fixed finger. The major clades a and b differed significantly in mean Von Mises stress and (size corrected) strain energy. As the Mann-Whitney test is based on ranking of the values, small datasets, such as the subclusters in this study, can be ordered in a limited number of ways. This leads to a tendency to converge on a small number of p-values, which accounts for the recurring value of some of the p-values in table 3. In addition, we also tested for differences in the angle between the inlever and the outlever of the movable finger between the clusters, but found no statistically significant differences.

Table 3. Mann-Whitney tests of statistical difference between named clusters of morphological and performance parameters. P-values higher than 0.05 are shown in grey font.

		a	b	a1	a2	a3	b1	b2
Chela height	a		0.052					
	b	1.1e−05						
	a1				0.057	0.057	**0.029**	0.067
	a2			0.057		0.700	0.057	0.714
	a3			0.057	0.100		0.057	0.905
	b1			**0.029**	0.057	0.057		0.114
	b2			**0.010**	**0.024**	**0.024**	**0.010**	
Movable finger length								
		a	b	a1	a2	a3	b1	b2
Mechanical advantage	a		2.06e−04					
	b	4.3e−05						
	a1				0.114	0.629	0.057	**0.010**
	a2			0.114		0.700	0.400	**0.024**
	a3			0.114	0.400		0.114	**0.024**
	b1			**0.029**	0.057	0.057		**0.019**
	b2			**0.010**	**0.024**	0.095	0.352	
		a	b	a1	a2	a3	b1	b2
Angle out-lever/Joint axis	a							
	b	1.30e−04						
	a1							
	a2			0.400				
	a3			0.057	0.700			
	b1			**0.029**	**0.050**	0.108		
	b2			**0.010**	**0.048**	0.095	0.109	
Chela aspect ratio								
		a	b	a1	a2	a3	b1	b2
Relative chela lengths	a		1.30e−04					
	b	0.218						
	a1				0.200	0.100	0.057	**0.024**
	a2			1.000		0.200	0.057	**0.048**
	a3			0.400	0.700		0.057	0.167
	b1			0.686	0.400	0.114		0.067
	b2			0.914	0.714	0.381	0.352	
Strain energy (corrected)								
		a	b	a1	a2	a3	b1	b2
Von Mises stress	a		4.3e−05					
	b	8.7e−05						
	a1				0.057	0.057	**0.029**	**0.010**
	a2			1.000		1.000	0.057	**0.024**
	a3			0.857	0.268		0.057	0.095
	b1			0.686	1.000	0.629		0.067
	b2			0.762	0.714	0.905	0.610	

Phylogenetic analysis and independent contrasts

The total aligned dataset consisted of 1534 positions for 20 species. Bootstrap support was high within the Buthidae, but interfamilial relationships could not be recovered with high support (not shown). Branch lengths were therefore calculated starting with a phylogeny with a polytomy uniting all family level clades (Figure 4).

The phylogenetic independent contrasts (Table 4) show both mean Von Mises stress and strain energy to correlate highly with several aspects of chela morphology, including size corrected height, width, outlever length, aspect ratio, mechanical advantage, and the ratio of the length of the movable finger to the distance between the joints. This corresponds with the observation from the clustering analysis that more elongate chelae experience higher stresses in the cuticula. Noteworthy is that the angle that the main axis of the movable finger makes with its axis of rotation is positively correlated with the length of the movable finger ("outlever" in Table 4), approaching a 90 degree angle for relatively longer fingers such as those of *Orthochirus*, *Hottentotta* and *Parabuthus*. Less surprising is that AR is highly correlated with the mechanical advantage of the chela. Neither mean Von Mises stress or strain energy correlated significantly with the angle between in- and outlever, the angle the outlever makes with the axis of rotation, or the curvature of the finger. This may indicate that these latter variables are independent from the performance of the chela in resisting stresses during pinching, and may be relevant for another function. The PIC analysis of the Buthidae only also showed high correlation coefficients for mean Von Mises stress and strain energy with aspect ratio, mechanical advantage and the ratio of finger length to the distance between joints EJ-MJ (0.79; 0.88; 0.91 respectively for both Von Mises stress and strain energy).

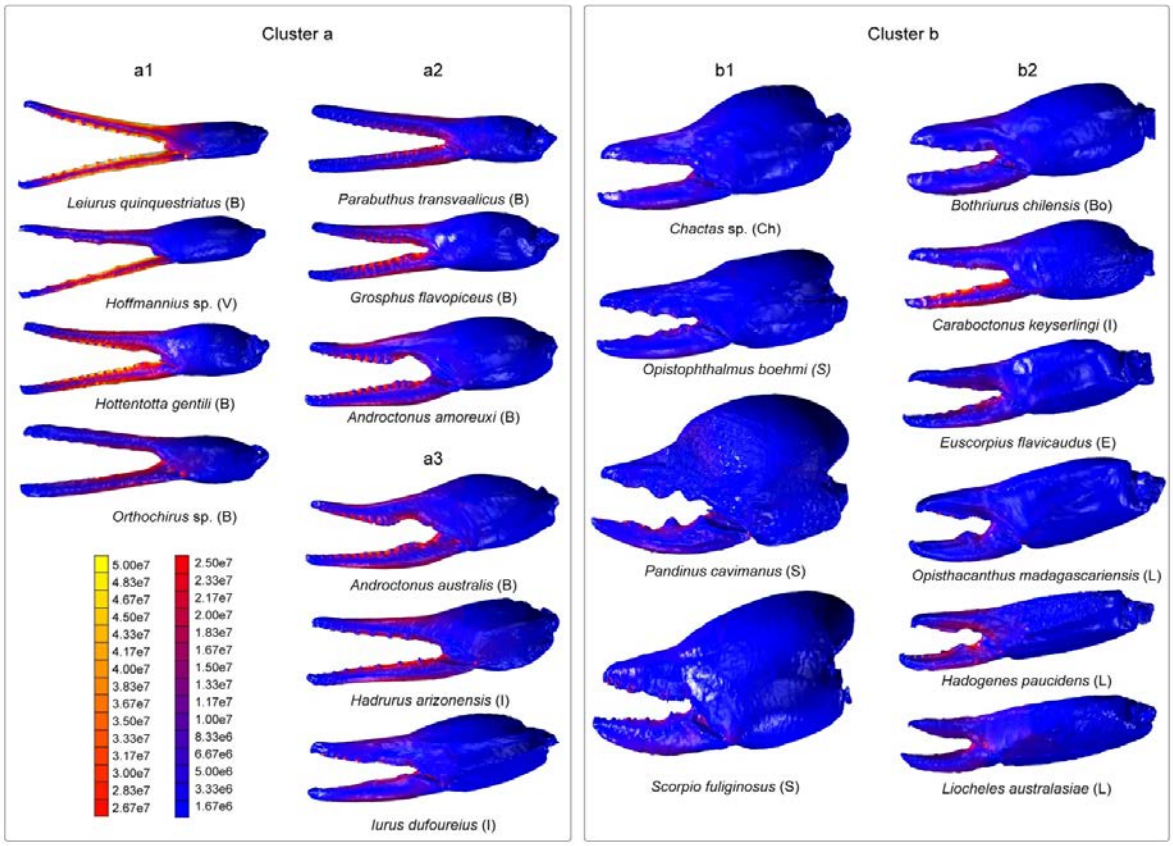

Figure 3. FEA models with calculated Von Mises stress visualized as colors. Despite producing much less force, chelae with a high aspect ratio and long fingers (cluster a), experience much higher stresses than the more robust chelae of cluster b. High deformation can be seen in *L. quinquestriatus*. High stresses indicate a risk of breakage, and the long-fingered chelae of cluster a are therefore at more at risk of breaking their fingers when exerting their maximum force.

Discussion

In order to provide a basis for comparing the FEA results for similarly shaped chelae, we carried out a cluster analysis. In the hierarchical cluster analysis, we identified clusters which corresponded in part with previously described ecomorphotypes. The major clusters differed significantly in stress and bending energy, with the more elongate chela forms experiencing much higher stresses and deformations (for which bending energy is a metric). Phylogenetic independent contrasts also show stress and deformation parameters to correlate highly with chela shape parameters. Despite the lack of a fully resolved molecular phylogeny for the taxa in this study, the PIC analysis strongly suggests chela shape and performance to correlate independently from phylogenetic history.

The clustering analysis revealed significant structure in the limited number of normalized measurements used. Although the expectation maximization algorithm found up to 8 clusters in the data, we chose a more conservative number of five clusters from the hierarchical cluster analysis. Several of the clusters roughly

corresponded to ecomorphotypes qualitatively described in the literature (Lamoral, 1979; Newlands, 1972; Polis, 1990), although these described ecomorphotypes are based on the whole body form, and not merely the chelae. The elongate chelae in cluster a, particularly those in cluster a1, correspond to the psammophylous ecomorphotype of Polis (1990). The taxa in this cluster indeed are all desert dwelling scorpions, although *Hottentotta gentili* tends to favor oases (Sousa et al., 2011). Also clusters a2 and a3 contain mostly species inhabiting dry areas, except for the Mediterranean *Iurus* and the Malagasy *Grosphus*. The species of clusters a2 and a3 are to a lesser extent psammophilous and/or vagrant. Cluster a is mostly comprised of species from the family Buthidae, with the exception of *Iurus*, *Hadrurus* and *Hoffmannius*. Since the Buthidae is the most basal family included in this study, we cannot conclude that these other taxa have evolved this chela morphotype independently or merely maintained the basal condition.

Custer b contains at least two distinct ecomorphotypes; the fossorial type (b1) and the infrasaxicolous and corticolous types (b2). The infrasaxicolous and corticolous types in cluster 2b have relatively shorter movable fingers. This may be due to the dorsoventral flattening that enables these species to live in the tight spaces between rocks, in rock cracks and under bark. A dorsoventral flattening of the chela would reduce the space available for muscle dorsally in the plane of the rotation of the movable finger. The bulk of the muscle is therefore placed more proximally, toward the base of the manus. The muscle filled part of the manus is relatively longer, conversely making the relative length of the movable finger shorter. The dorsoventral restriction also reduces the possible length of the inlever of the movable finger, which may explain the difference ($p=0.035$; table 3) in the mechanical advantage between cluster b1 and b2. The fossorial species comprising cluster b1 are not limited dorsoventrally, and have the highest chelae, with a mechanical advantage favoring high forces. Despite that such a lever system would reduce closing speed if muscle fibers contract at a constant rate (Arnold et al., 2011), this may not make these scorpions slower in closing their chelae. Since the high chelae allow their muscle fibers to be much longer, and longer muscle fibers contract over a longer distance than shorter ones per unit of time, this effect may hypothetically partly offset the reduction in closing speed caused by the mechanical advantage of the lever system. The scorpions in cluster b1 are mostly fossorial species, which use their strong chelae for burrowing in hard soil. The scorpions of cluster b2 do not need their relatively strong chelae to dig, but it has been suggested that some members of this clade are durophagous. Particularly *Hadogenes* has been known to prey upon hard-shelled prey such as millipedes and even snails (Newlands, 1978).

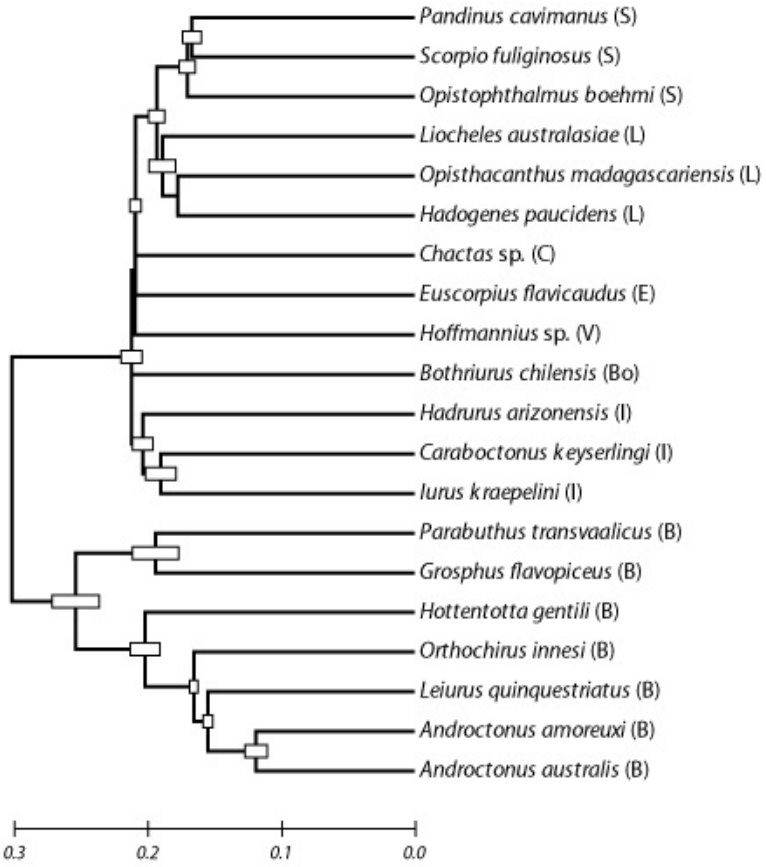

Figure 4. Linearized tree with branch lengths based on 12S, 16S and CO1 sequences. Phylogenetic independent contrast calculations (table 4) were based on this phylogram.

The mean Von Mises stress is significantly lower in the more robust chelae morphologies comprising cluster b. This indicates that under maximal defensive loadings, these chelae are less likely to fail than those of cluster a. The same pattern can be seen for the strain energy, indicating that these chela shapes also deform less. The size-corrected FEA analysis allows the comparison of the effect of shape alone on chela performance. Some of the more extreme chela shapes differ highly in performance; the bending energy in *Leiurus* is nearly 100 times higher than that in *Scorpio*, and the mean stress more than 10 times higher. These differences can be attributed to shape alone, making chela shape a very important factor in the performance of the chela. In order to make models comparable and isolate the effect of shape, the material characteristics (Young's module and Poisson's ratio) of the cuticula were set to be the same in all species. This assumption may well be idealistic, as even within a single specimen, cuticula hardness is known to vary widely (Schofield, 2001). Due to application of the total load in several point loads, local stress may have become artificially high. This consideration necessitated us to disregard maximum Von Mises stress as a performance variable.

Pearson correlation coefficients		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Mean Von Mises stress		<1e−4	0.158	0.123	0.001	<1e−4	0.014	<1e−4	<1e−4	<1e−4	0.280	0.557	0.072
2	Strain energy	**(0.96)**		0.542	0.472	<1e−3	<1e−4	0.010	<1e−4	<1e−4	<1e−4	0.126	0.719	0.056
3	Absolute prosoma length	0.35	0.15		<1e−4	0.806	0.946	0.520	0.708	0.696	0.913	0.429	0.985	0.502
4	Absolute chela length	0.38	0.18	**0.92**		0.482	0.759	0.718	0.707	0.599	0.909	0.162	0.556	0.300
5	Standardized width	**−0.71**	**−0.74**	−0.06	−0.18		<1e−4	0.339	<1e−3	**0.001**	**0.003**	0.167	0.792	0.568
6	Standardized height	**−0.84**	**−0.91**	0.02	−0.08	**0.85**		0.096	<1e−3	<1e−4	<1e−4	**0.044**	0.807	0.200
7	Standardized out-lever length	**0.57**	**0.59**	0.16	0.09	−0.24	−0.4		**0.020**	**0.001**	<1e−3	0.122	0.094	**0.005**
8	Aspect ratio chela	**0.81**	**0.89**	0.1	0.1	**(−0.78)**	**(−0.84)**	**0.54**		<1e−4	<1e−4	0.162	0.486	0.173
9	Mechanical advantage	**0.9**	**0.94**	0.1	0.13	**−0.71**	**−0.87**	**(0.72)**	**0.89**		<1e−3	0.099	0.442	**0.027**
10	Ratio finger length/ distance LJ-MJ	**0.83**	**0.92**	−0.03	−0.03	**−0.67**	**−0.84**	**(0.75)**	**0.86**	**(0.94)**		**0.039**	0.186	**0.003**
11	Curvature of movable finger	0.27	0.37	−0.2	−0.34	−0.34	**−0.48**	0.38	0.34	0.4	0.49		0.856	0.411
12	Angle in-lever/out-lever	−0.15	−0.09	0	0.15	0.07	−0.06	−0.41	−0.18	−0.19	−0.33	−0.05		<1e−3
13	Angle out-lever/axis of rotation	0.43	0.46	−0.17	−0.26	−0.14	−0.32	**0.63**	0.34	**0.52**	**(0.66)**	0.21	**−0.74**	

Table 4. Phylogenetic independent contrasts. Values below the diagonal are Pearson correlation coefficients, values above the diagonal are the corresponding p -values. Correlation coefficients with a p-value < 0,05 are marked in bold. P-values above 0,05 are in grey font. Correlation coefficients that result from correlation of non-independent variables are in brackets. All non-ratio and non-angle values were log-10 transformed.

The lack of a robust molecular phylogenetic hypothesis for the high-level relationships between scorpions makes it hard to make any statements about the independent evolution of ecomorphotypes. It is well known that chela shape can vary widely within scorpion families, such as the Buthidae and Vaejovidae (Stockmann & Ythier, 2010). Also in our clustering analysis, members of the same family (Iuridae) were present in different clusters. This suggests chela shape may well be a homoplastic character. Since changes in the overall shape of the chela may change the relative position of taxonomic characters (Prendini, 2000), systematists using morphological characters of the chela, such as the relative positions of the trichobothria, may need to test for the independence of their characters to the overall chela shape parameters which correlate highest with performance, such as height, width and relative finger length. Since only relatively fast-evolving mitochondrial genes were used to infer branch lengths, the branch lengths used to calculate the phylogenetic independent contrasts may be an underestimate of the rate of evolution in the basal part of the tree. However, the PIC method analysis has been shown to be a robust method in relation with branch length distributions (Diaz-Uriarte & Garland, 1996; Ackerly, 2000).

Concluding remarks

Our work presents the first mechanical models for the computational assessment of performance of the scorpion chelae, and allows chela shape types to be used as an approximation for performance. Despite the limited number of taxa included, our results clearly show that more elongate chelae experience higher stresses and deformations than more robust chelae. This makes these chelae less suitable for tasks that require the chela to perform near its maximum, such as defense, subduing of hard prey, and burrowing. This may be the reason why scorpions with slender chelae use their sting more in the incapacitation of prey (Stahnke, 1966; McCormick & Polis 1990).

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Chapter IV

Final Remarks and Future Research
References

4.1 Final Remarks

From an original plan to sequence nuclear genes we faced technical difficulties at different levels in the whole process. Initially, we tried to amplify 18S and 28S rRNA genes in order to obtain nuclear DNA. However, analysis of the dataset produced by the resulting sequences led to the conclusion that these genes have been duplicated in our study organisms, or at least that concerted evolution was not causing the sequences to be identical but rather two divergent groups occurred within individuals. Unfortunately, this event made it impossible to infer phylogenetic patterns and, consequently, this dataset had to be discarded. Then, our attention turned to mitochondrial DNA (mtDNA) in order to sequence phylogenetically-informative DNA that could be used not only at the genus level, but also at the sub-family level. Cytochrome b (Cytb) was first selected as it is routinely used in phylogenetic studies. However we faced technical difficulties trying to amplify this fragment. As time consuming as it was, we felt obligated to choose other mitochondrial genes that could produce the whole dataset quicker. Therefore, we chose the 12S rRNA, 16S rRNA and COI genes. We were able to produce the whole dataset with less than 10% of missing data. In the middle of the process, we made other attempts to use phylogenetic informative genes at different resolutions inside the Scorpiones order. Histone 3 (H3) was part of the laboratorial work produced in 2011/2012 that was chosen based on its wide use in phylogenetic studies of the Aranae order. Like CytB it showed sub-standard amplification. The last attempt fell upon ND1 mtDNA gene and, although it had low amplification success, it was informative. In addition, it is a protein coding gene like COI. In a period where we already had two non-coding and two protein-coding genes we made a final but unsuccessful endeavor to amplify informative nuclear genes. We used primers for six nuclear genes from Gantenbein and Eightley, 2004, using a total of eight primer combinations. As we could not successfully amplify these last genes we chose to complete our studies with the 16S + 12S + COI or ND1 dataset.

4.2 Future Research

Technical obstacles played against the original goal of obtaining phylogenies that could resolve relationships within the Scorpiones order, between families, genera and species with strong resolution. A lack of slower evolving nuclear markers hampered basal inference on the *Androctonus* and the scorpion families' phylogenies. Future work should be guided towards the improvement of DNA markers that can produce well resolved phylogenies within different scorpions. The effect of model choice (Jones *et al*., 2006) and testing for recombination (Gantenbein et al., 2005) should be taken into consideration in future studies as well.

Additionally, new strategies must be created to overcome sampling difficulties in traditionally inaccessible countries. Political instability on one hand and the lack of georeferenced sampling on the other might contribute to an underestimation of scorpion' diversity levels.

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